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β-1,6-glucan plays a central role in the structure and remodeling of the bilaminate fungal cell wall

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eLife Assessment

The paper will be of broad interest to fungal biologists and fungal immunologists seeking to understand the biosynthesis of the fungal cell wall, in particular of ß-1,6-glucan synthesis and the importance of this so far understudied constituent of the cell wall for cell wall integrity and immune response. The study is of **fundamental** significance and adds structural clarity to the genetic, and biochemical basis of this difficult-to-analyze carbohydrate. It opens the potential for understanding its role in immune recognition and potentially as a drug target. Overall, the data is **compelling**, properly controlled and analyzed.

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Abstract

The cell wall of human fungal pathogens plays critical roles as an architectural scaffold and as a target and modulator of the host immune response. Although the cell wall of the pathogenic yeast *Candida albicans* is intensively studied, one of the major fibrillar components in its cell wall, β-1,6- glucan, has been largely neglected. Here, we show that β-1,6-glucan is essential for bilayered cell wall organization, cell wall integrity and filamentous growth. For the first time, we show that β-1,6- glucan production compensates the defect in mannan elongation in the outer layer of the cell wall. In addition, β-1,6-glucan dynamics are also coordinated by host environmental stimuli and stresses with wall remodeling, where the regulation of β-1,6-glucan structure and chain length is a crucial process. As we point out that β-1,6-glucan is exposed at the yeast surface and modulate immune response, β-1,6-glucan must be considered a key factor in host-pathogen interactions.

Introduction

Pathogenic fungi are a significant burden to public health, recently estimated to cause 2.5 million annually attributable deaths worldwide[1]. Among these, *Candida* species are responsible for more than 100 million mucosal infections and 1.5 million systemic infections, representing the fourth most frequent cause of bloodstream infection in hospitalized patients[1–3]. *Candida albicans* is the most common opportunistic species, which in healthy individuals often colonizes mucosal surfaces (oral cavity, gastrointestinal tract, as well as genital tract)[4], but leads to invasive infection, post trauma or when immune system of the host is compromised. *C. albicans* is classified in the critical priority group of the WHO list of fungal pathogens[5].

Fungal cells are enveloped by a cell wall, which is essential for protection, integrity, and to maintain cell morphology. During commensalism with human hosts, the cell wall enables fungi to survive host defenses, whereas during pathogenic interactions, the cell wall also plays a central role in the polarized growth of invasive hyphae, thereby facilitating host tissue invasion[6,7]. The cell wall architecture is dynamic and therefore critical in modulating the host immune responses[8,9], which are driven by host immune receptors (pattern recognition receptors [PRRs])[10–12]. Recognition of fungal pathogen-associated molecular patterns (PAMPs) by host PRRs triggers an array of antifungal responses, including phagocytosis, the production of reactive oxygen/nitrogen species (ROS/RNS), neutrophil extracellular traps, and the release of cytokines and chemokines that recruit immune cells to the site of infection and activate adaptive immunity[12–15]. Being extracellular, thereby first to interact with the host immune system, fungal cell wall components function as the major PAMPs.

The cell wall of *C. albicans* is a complex structure with a fibrillar outer layer of mannoproteins and an inner polysaccharide layer composed of chitin, β-1,3-glucan and β-1,6-glucan, which are not present in mammalian cells[16]. Dectin-1, a C-type lectin receptor that recognizes fungal cell wall β- 1,3-glucan, has been demonstrated to play a dominant pro-inflammatory role in antifungal immunity[17–21]. Chitin particles derived from the *C. albicans* cell wall lead to the selective secretion of the anti-inflammatory cytokine IL-10[22]. Mannans from *C. albicans* are recognized by multiple receptors expressed on different immune cells (Mannose receptor, DC-SIGN, TLR4, Dectin-2, Dectin-3), which are involved in yeast phagocytosis and appear to play a critical role in the induction of Th17 responses[23,24]. *C. albicans* has developed strategies to sense environmental signals encountered within the host and to regulate the exposure of its cell wall PAMPs. For example, low levels of β-1,3-glucan exposure by *C. albicans* correlate with enhanced colonization of the gastrointestinal tract[25,26] and β-1,3-glucan is mainly hidden from immune recognition by the outer layer of mannan fibrils[11,27,28]. Although these *C. albicans* cell wall polysaccharide PAMPs have been investigated in the context of host-fungal interaction, the immunomodulatory potentials of β-1,6- glucan, another major constituent of the *C. albicans* cell wall, remains under-investigated.

The composition and organization of the fungal cell wall result from an equilibrium between syntheses and remodeling of the constituent polysaccharides[23,29]. β-glucans and chitin, which account for about 60% and 2-10% (depending on the morphotype), respectively, are cross-linked to form the skeletal armor of the *C. albicans* cell wall[15,30]. Mannoproteins, which are predominantly glycosylphosphatidylinositol (GPI)-modified and covalently linked to the skeletal polysaccharides via β-1,6-glucan bridges[30,31], account for 30%–40% of the cell wall. β-1,6-glucan is composed of a linear chain of β-1,6-linked glucose residues with few short side-chains of β-1,3-glucoside or laminaribioside residues[32,33]. According to analytical methods, the β-1,6-glucan content is highly variable, from 15 to 50%[34–37]. Although β-1,6-glucan plays a crucial structural role in the *C. albicans* cell wall, the dynamics of this cell wall polysaccharide as a function of growth or stress conditions remains underexplored.

Genes required for the full β-1,6-glucan production have been identified in model yeast, *Saccharomyces cerevisiae*[38]. Among them, *KRE5* codes a putative α-glucosyltransferase[39] (GT-24) localized in the endoplasmic reticulum (ER); its deletion results in the absence of β-1,6-glucan in *S. cerevisiae* and about 80% reduction of cell wall β-1,6-glucan content in *C. albicans*[35,40]. Functions of Kre1, Kre6, Kre9 and the related homologs are unclear. Their localization in the ER, Golgi, plasma membrane or extracellular culture medium, suggests an intracellularly initiating biosynthetic pathway of β-1,6-glucan[41,42]. *KRE6* and its functional homolog *SKN1* share 66% identity and both encode transmembrane proteins containing a glycosyl-hydrolase domain (GH-16). *KRE6* deletion in *S. cerevisiae* results in a 70% decrease in the cell wall β-1,6-glucan content[33]. In *C. albicans* the double deletion of *KRE6* and *SKN1* leads to a slow growth, cell wall abnormalities, cell separation failure, and reduced virulence[43,44]. Two more *KRE6* homologs (*KRE62* and *SKN2*) have been identified in *C. albicans*[45] but their function has not yet been investigated, and the *C. albicans* cell wall β-1,6-glucan biosynthetic pathway, namely the β-1,6-glucosyltransferase involved in β-1,6- glucan polymerization and other enzymes involved in β-1,6-glucan remodeling (elongation, branching or cross-linking with β-1,3-glucan) remains to be identified.

We standardized biochemical approaches to quantify as well as to characterize β-1,6-glucan of *C. albicans*, which allowed us to perform comparative analysis and to study the dynamic of this cell wall polysaccharide in response to stress conditions and to antifungal treatments. β-1,6-glucan biosynthesis appears to be a compensatory reaction when mannan elongation is defective, whereas the absence of β-1,6-glucan results in a significantly sick growth phenotype and complete cell wall reorganization. Moreover, β-1,6-glucan showed a distinct immunomodulatory response compared to other cell wall polysaccharides. Together, our study establishes overarching and critical roles for β- 1,6-glucans in the organization, structure, biosynthesis and immunomodulatory properties of the cell wall.

Results

β-1,6-glucan - a critical polysaccharide of *C. albicans* cell wall

Despite numerous studies[34–37], the composition and organization of *C. albicans* cell wall β-1,6-glucan remain unclear. To better understand the composition and organization of the cell wall β-1,6-glucan, we have fractionated cell wall and applied new biochemical strategies to quantify and characterize them. The *C. albicans* cell wall was separated into three fractions according to polymer properties and reticulations, namely: sodium-dodecyl-sulphate-β-mercaptoethanol (SDS-β-ME) extract, alkali- insoluble (AI) and alkali-soluble (AS) fractions (see methods). In Synthetic Dextrose (SD) medium at 37°C, the SDS-β-ME fraction was the smallest (18% of total cell wall) and mainly composed of mannoproteins (**Fig. 1a**). The AI fraction was the major one (46%) and was exclusively composed of glucose and glucosamine, the monosaccharide building blocks of β-glucans and chitin, respectively. The AS fraction (36% of total cell wall) was characterized by a high amount of mannose residues (54% of the fraction) resulting from the release of covalently cell wall-anchored mannoproteins (**Fig. 1a**), and this fraction also contained glucose residues originating from β-glucans. Overall, we found that the *C. albicans* cell wall is composed of 3% of chitin, 43% of proteins/mannoproteins (27% of mannans) and 54% of β-glucans, which was in agreement with earlier studies and validated our analytic approach (Fig.1a, 1b)[46–48].

To differentiate β-1,3-glucans and β-1,6-glucans, we developed two methods, one for the AI fraction and other for the AS fraction (see methods, and **Fig. S8**) as β-glucan was found in both fractions. β- 1,6-glucan corresponded to 23% of the total cell wall, was mainly found in the AI fraction (**Fig. 1a**, 1b). β-1,6-glucan in the AI fraction could be solubilised by digesting β-1,3-glucan using an endo-β- 1,3-glucanase, confirming that it is covalently linked to the cell wall β-1,3-glucan as previously shown in *S. cerevisiae*[33,49]. Further, gel filtration chromatography showed that the

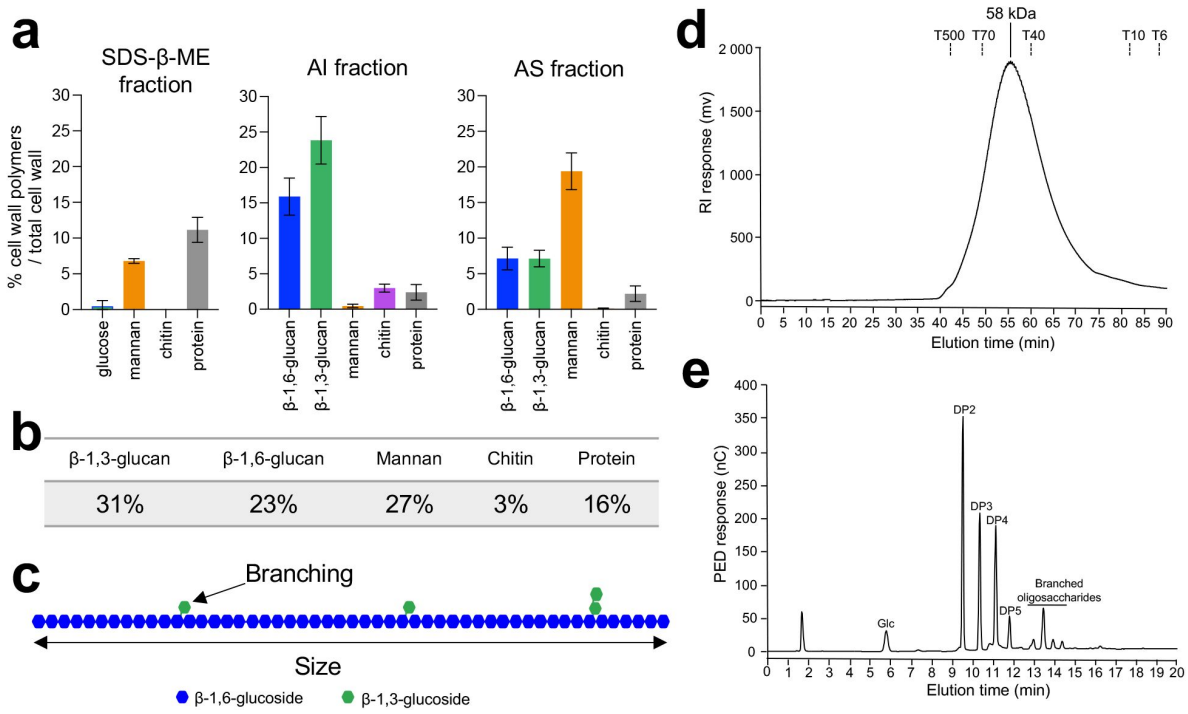

Figure 1

Analysis of *C. albicans* cell wall β-1,6-glucans.

a, Percentages of cell wall polymers on total cell wall, distributed by fractions: SDS-β-ME, AI and AS. Cells were grown in SD medium at 37°C. Means and standard deviations were calculated from three independent experiments. **b**, Table of the mean percentages of each polymer in the cell wall from three independent experiments. **c**, Diagram of β-1,6-glucan structure. In blue are represented glucose residues linked in β-1,6 and in green glucose residues linked in β-1,3. According to NMR analysis and HPAEC after endo-β-1,6-glucanase digestion (**Table S2**), based on three independent experiments, an average of 6.4% (+/- 0.5%) of glucose units of the main chain are substituted by a single glucose residue (88-90%) or a laminaribiose (10-12%). **d**, Gel filtration analysis on a Superdex 200 column of β-1,6-glucan released by endo-β-1,3-glucanase digestion. The column was calibrated with dextrans (Tx: x kDa). **e**, HPAEC chromatographic analysis of the digestion products of the AI fraction treated with an endo-β-1,6-glucanase. Chromatographs in d and e are representative of three independent experiments. *PED*, pulsed electrochemical detector; *nC*, nanocoulombs; *RI*, refractive index; *mV*, millivolt; DP, degree of polymerization; Glc, glucose.

solubilised β-1,6-glucan was eluted in a single symmetric peak with Gaussian shape showing a homogeneous polymer with an apparent molecular weight range of 18-112 kDa (**Fig. 1d**), and the average molecular weight was estimated to be 58 kDa. The structure of purified β-1,6-glucan was analysed by ^{1}H and ^{13}C-NMR (**Table S2**) and by HPAEC after endo-β-1,6-glucanase digestion (**Fig. 1e**). The NMR chemical shifts and coupling constants as well as the HPAEC profile of the digested products were highly comparable to those we described for *S. cerevisiae*[33], showing a similar structure: a main chain of β-1,6-glucosyl residues with side chains of β-1,3-glucosyl or β-1,3-laminaribiosyl residues. The relative intensity of the anomeric proton in NMR and HPAEC analysis after endo-β-1,6-glucanase digestion, indicated that an average of 6.4% of glucose units of the main chain is substituted and that 88-90% of the side chains is composed of a single glucose residue (**Fig. 1c, 1e**, **Table S2**). Altogether, our data showed that β-1,6-glucan is a major cell wall polymer in *C. albicans,* representing 40% of the cell wall β-glucans.

Environmental regulation of β-1,6-glucan synthesis

The composition and surface exposure of fungal cell wall polymers is dependent on environmental growth conditions[26,29]. We applied chemical assays and our biochemical approaches to enable comparative analyses of cell wall robustness in relation to β-1,6-glucan dynamics in *C. albicans*.

First, we analysed the cell wall composition in relation to cell morphotype and growth phase (exponential vs. stationary phase, yeast vs. hyphae, planktonic vs. biofilm). No significant difference was observed between cell wall β-1,6-glucan and other polymers produced during the exponential (control) and stationary phases in yeast cells (**Fig. 2**). In biofilms, only a slight increase of mannan content (26% vs. 20%), but no change in relative β-glucan content was observed. In contrast, the cell wall isolated from *C. albicans* hyphae was characterized by an increase in chitin content (10% vs. 3%) as previously shown[29,48,50], as well as a reduction of β-1,6-glucan (18% vs. 23%) and mannan contents (13% vs. 20%) compared to the yeast form. The decrease in β-1,6-glucan content was reflected in a reduction of the β-1,6-glucan size (43 vs. 58 kDa), demonstrating that both content and structure of β-1,6-glucan are regulated during growth and cellular morphogenesis. In addition, a higher branching rate of β-1,3-glucan of the hyphal cell wall was also observed in the AI fraction (7.3% vs. 5.3%) (**Fig. S3**).

We further investigated the effect on cell wall composition of environmental growth conditions, such as temperature, pH, carbon source and hypoxia, on cell wall composition (**Fig. 2**). Hypoxic condition (1% O2, 5% CO2) resulted in a slow growth but did not lead to a significant alteration of the cell wall composition. In contrast, all other tested conditions led to a modification of cell wall composition including the proportion of β-1,6-glucan. For example, growth at 30°C or in 1M NaCl led to a decrease in the cell wall β-1,6-glucan content and a decrease of β-1,6-glucan chain length. However, these decreases at low temperature were compensated by a higher content of less-branched β-1,3- glucan, whereas high salt led to an increased branching rate of both β-1,6-glucan and β-1,3-glucan (**Fig. 2**, Fig.S3). Buffered culture medium at pH 4 or 7.5 led to a slight decrease in the β-1,6-glucan molecular weight and an increase in its branching frequency in comparison to buffered medium at pH 5.4. However, the most pronounced alteration of the cell wall was observed in lactate containing medium, with a steep reduction of polysaccharides from the structural core, chitin (−43%), β-1,3- glucan (−48%) and mainly β-1,6-glucan (−72%), in agreement with the decrease in thickness of the inner cell wall layer previously described[51]. Lactate medium also led to a strong reduction in the average β-1,6-glucan molecular weight (19 vs. 58 kDa), a decrease of β-1,6-glucan branching (4.9 vs. 6.4%) and an increase of β-1,3-glucan branching (7.4 vs. 5.3%, **Fig. S3**).

The presence in the medium of short chain fatty acids (acetate, propionate, or butyrate) at physiological gut concentrations resulted in a growth defect but did not alter the global cell wall composition. The main difference was an increase of β-1,6-glucan molecular weight (64-68 kDa vs. 58 kDa) (**Fig. 2b**). Oxidative stress and drugs targeting the cell wall did not induce any major

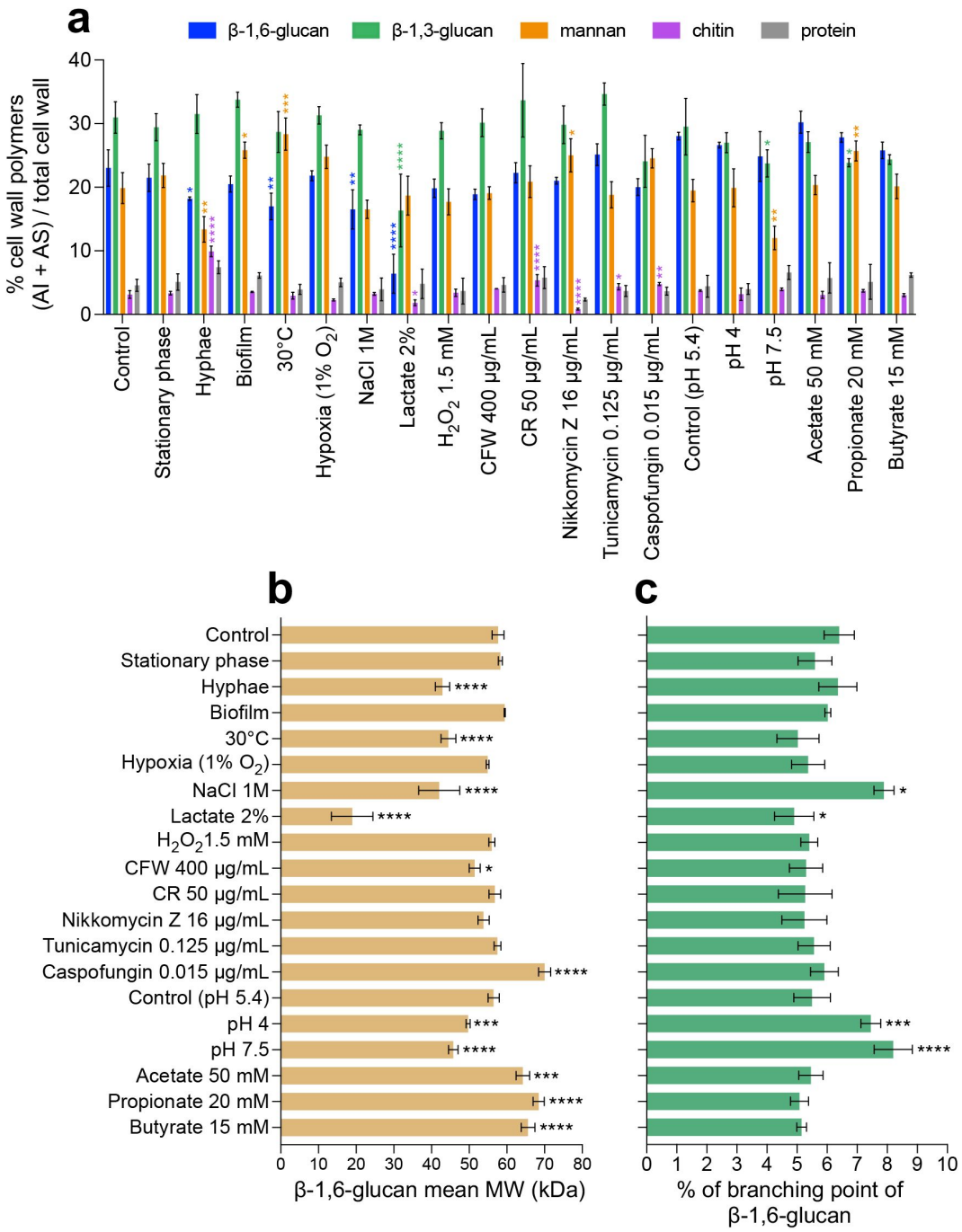

Figure 2

Comparative analyses of cell wall β-1,6-glucans produced in various environmental conditions.

a, Percentages of cell wall polymers (AI + AS fractions) on total cell wall. Cells were grown in liquid synthetic medium at 37°C under different conditions, as specified in Methods. **b**, β-1,6-glucan mean molecular weight (MW). Average molecular weight was estimated by gel filtration chromatography on a Superdex 200 column. **c**, Branching rate of β-1,6-glucans. Branching rate was determined by HPAEC after digestion of the AI fraction by an endo-β-1,6-glucanase (% expressed as number of glucose units of the main chain that are substituted by a side chain). For a, b, and c, means and standard deviations from three independent replicate experiments are shown. All data were compared to the control conditions and were analysed by one-way ANOVA with Dunnett's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$.

alterations (**Fig. 2**). Cell wall chitin content was reduced in the presence of nikkomycin, a chitin synthesis inhibitor, and increased in the presence of the cell wall damaging agents Congo Red or caspofungin, as previously observed[52–54], but with no impact on β-1,3-glucan and β-1,6-glucan contents. The major stress effect we observed was in presence of caspofungin, which caused an increase of the average molecular weight of β-1,6-glucan (70 vs. 58 kDa).

These data demonstrate that the content, size, and branching of β-1,6-glucan in the *C. albicans* cell wall is regulated by numerous environmental growth and stress conditions.

β-1,6-glucan dynamics responds to alterations in inner cell wall synthesis

We investigated the dynamics of β-1,6-glucan in *C. albicans* mutants in which the genes involved in the biosynthesis or remodelling of chitin and β-1,3-glucan were deleted. Four chitin synthases, located at the plasma membrane, have been described in *C. albicans* and we have analysed three deletion mutants. Chs1 is involved in primary septum formation and is essential for vegetative growth. We therefore analyzed the conditional P*MRP1-CHS1/chs1*Δ mutant under repressive condition. Chs2 is a non-essential class II enzyme and represents the major chitin synthase activity measured in cell membrane preparations[55]. Chitin synthase 3 (Chs3) is responsible for most of the cell wall chitin production and chitin ring formation at sites of cell division[56] but it is not essential for growth and viability. Our comparative analyses did not show any alteration in the chitin content in the P*MRP1-CHS1/chs1*Δ and *chs2*Δ/Δ mutants, but a strong reduction was observed in the *chs3*Δ/Δ mutant (−92.3%) (**Fig. S2**) in accordance with previous data[57,58]. In the P*MRP1-CHS1/chs1*Δ mutant, the cell wall had a 45.7% decrease in mannan content in the AS fraction and a 50% increase in β- 1,6-glucan in the AI fraction (**Fig. S2**, **Fig. 3a**). In the *chs3*Δ/Δ mutant, the low proportion of chitin led to an increase of both β-1,3-glucan (x1.6) and β-1,6-glucan (x1.6) in the AS fraction (**Fig. 3a**, **Fig. S2**), suggesting a defect in β-glucans cross-linking in the cell wall polysaccharide core. No significant change in β-1,6-glucan structure was noticed for these two mutants (**Fig. 3b**, **3c**). Strikingly, no significant effect of the *CHS2* deletion was observed on cell wall chitin content or overall wall composition.

FKS1, *FKS2* and *FKS3* encode β-1,3-glucan synthases in *C. albicans*[48,59]. As *FKS1* is essential for vegetative growth[59], we used the heterozygous *fks1*Δ mutant, which displays a haplo-insufficiency phenotype[60]. Only a decrease (−58.7%) in β-1,3-glucan content in the AS fraction (**Fig. S2**) was observed, without any other significant changes. Moreover, essentiality of *FKS1* prevented analysis of β-1,6-glucan dynamics in a β-1,3-glucan-deficient mutant. *PHR1* and *PHR2* encode β-1,3- glucanosyltransferases of the GH-72 family involved in β-1,3-glucan elongation and branching at the extracellular cell wall level[61,62]. In *C. albicans* their expression is strongly pH dependent: *in vitro*, *PHR1* is expressed above pH 5.5, while *PHR2 is* expressed below pH 5.5[63]. The *phr1*Δ/Δ mutant was thus grown at pH 7.5 and *phr2*Δ/Δ at pH 5.5. As expected for both mutants, defects in GH-72 β-1,3-glucan remodelase led to a decrease in β-1,3-glucan branching (−67.3% for *phr1*Δ/Δ, −31.5% for *phr2*Δ/Δ) (**Fig. S4**) and a compensatory increase in the cell wall chitin content (1.5x for *phr1*Δ/Δ, and 3.7x for *phr2*Δ/Δ) (**Fig. S2**). No change in the cell wall β-1,3-glucan content was observed in the *phr1*Δ/Δ mutant (**Fig. S2**). In contrast, deletion of *PHR2* led to an increase of β-1,3-glucan content and a cross-linking defect (−31.5% estimated on the ratio β-1,3-glucan content in AS and AI fractions). Concomitantly, deletion of *PHR2* led to a reduction of cell wall β-1,6-glucan content in both AI (−58.7%) and AS (−51.3%) fractions and to a reduction of molecular weight and branching of the β-1,6-glucan chains (**Fig. 3a**). In the *phr1*Δ/Δ mutant, only a significant increase of the molecular weight of β-1,6-glucan chain was observed (60 vs. 50 kDa) (**Fig. 3**).

Taken together, these data showed that mutational disruption of chitin-synthase or β-1,3-glucan-synthase genes did not significantly alter the cell wall β-1,6-glucan structure. However, defects in chitin content led to a decrease in the cross-linking of β-glucans in the inner wall (i.e. AI Fraction)

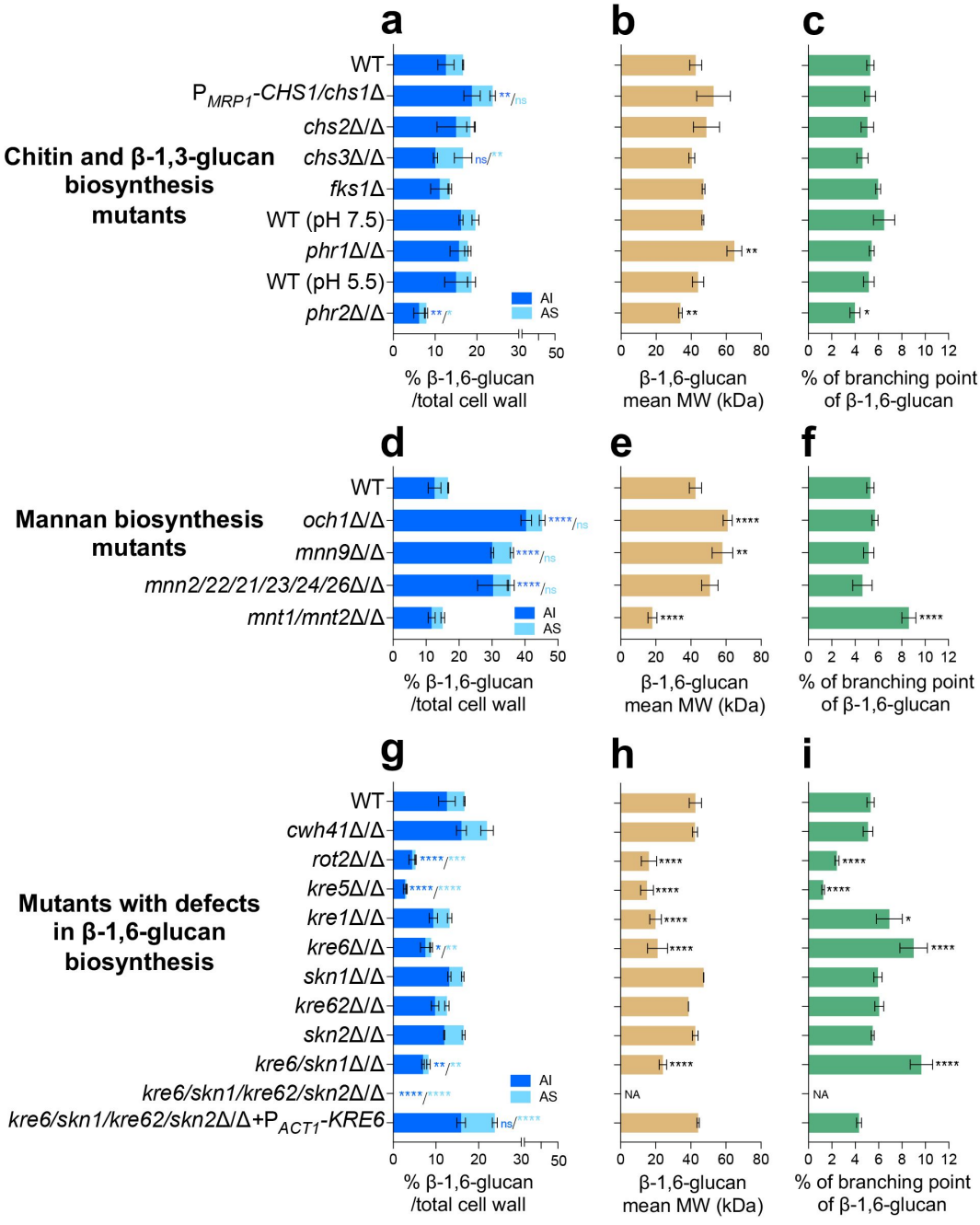

Figure 3

Comparative analysis of β-1,6-glucan content and structure produced by cell wall mutants.

a, **d, g** Percentages of cell wall β-1,6-glucans (AI and AS fractions) on total cell wall. **b**, **e**, **h**, β-1,6- glucans mean molecular weight (MW) and **c**, **f**, **i** Branching rate of β-1,6-glucans. Cells were grown in liquid SD medium at 30°C. Means and standard deviations from three independent replicate experiments are shown. All data were compared to the control conditions and were analysed by one-way ANOVA with Dunnett's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$; ns: non-significant; NA: non-applicable.

that corresponds to the effect of nikkomycin treated *C. albicans* phenotype (**Fig. S1**); conversely, an increase in chitin content led to more cross-linking of β-glucans as observed in the *FKS1* mutant or in the presence of caspofungin (**Fig. S1** and **S2**). In contrast, disruption of cell wall β-1,3-glucan remodelling had a pH-dependent effect on β-1,6-glucan structure (**Fig. 2** and **3**).

A β-1,6-glucan mannan compensatory pathway links the regulation of the inner and outer cell wall layers

In *C. albicans*, mannosylation of cell wall proteins is required for the organization of the fibrillar outer layer of the cell wall that is composed of *N*-linked mannans[27,64]. This fibrillar layer is anchored to the polysaccharide core composed of β-1,3-glucan and chitin, via β-1,6-glucan bridges[30]. We analyzed mutants deficient in Golgi α-mannosyltransferases involved in *O*- and *N*-mannosylation. Mnt1 and Mnt2 are α-1,2-mannosyltransferases involved in catalyzing consecutive reactions in *O*-mannan elongation[23,65]. As predicted, the cell wall of the *mnt1/mnt2*Δ/Δ mutant showed minor changes in overall mannan content (**Fig. 3d**, **Fig. S2**)[65]. However, deletion of *MNT1* and *MNT2* genes led to a strong reduction of β-1,6-glucan molecular weight (18 vs. 43 kDa) and an increased branching of β- 1,6-glucan chains (8.6 vs. 5.3%) (**Fig. 3e**, **3f**). A slight increase in the β-1,3-glucan branching was also observed in the *mnt1/mnt2*Δ/Δ double mutant (**Fig. S4**). Overall, these data suggested that β- 1,6-glucan and mannan syntheses may be coupled.

N-mannan elongation requires the activity of a set α-1,6-mannosyltransferases in the Mannan Polymerase I and II complexes for the formation of α-1,6-mannan backbone that is subsequently modified with α-1,2- and α-1,3-mannosyltransferases that assemble the *N*-mannan side chains[23]. Och1 activity is responsible for the initial addition of α-1,6-mannose onto the relatively well conserved *N*-glycan triantennary complex of glycoproteins, and Mnn9 activity is essential for the polymerization of the α-1,6-mannose backbone. Mnn2, Mnn21, Mnn22, Mnn23, Mnn24 and Mnn26 activities are essential to the synthesis of side chains[23]. Single *och1*Δ/Δ and *mnn9*Δ/Δ mutants and the sextuple *mnn2/21/22/23/24/26*Δ/Δ mutant resulted in a major decrease in mannan content in the AS fraction (−90.4% for *och1*Δ/Δ, −88.9% for *mnn9*Δ/Δ and −87.1% for *mnn2/21/22/23/24/26*Δ/Δ) and a compensatory increase in chitin content (x3.1 for *och1*Δ/Δ, x2.5 for *mnn9*Δ/Δ and x2.5 for *mnn2/21/22/23/24/26*Δ/Δ) in the AI fraction (**Fig. S2**), as previously described[23]. A significant increase of cell wall β-1,3-glucan content was observed in the *mnn9*Δ/Δ and *mnn2/21/22/23/24/26*Δ/Δ mutants. There was also a significant increase in the β-1,6-glucan content (x3.2 for *och1*Δ/Δ, x2.4 for *mnn9*Δ/Δ and x2.4 for *mnn2/21/22/23/24/26*Δ/Δ respectively) concomitant with the elongation of β-1,6-glucan chains, and consequential increase in its molecular weight (**Fig. 3d**, **3e**).

Overall, these data suggest that the β-1,6-glucan biosynthesis is stimulated via a compensatory pathway when there is a defect in *O*- and *N*-linked cell wall mannan biosynthesis. This novel compensatory response occurs by increasing the β-1,6-glucan content in *N*-mannan elongation-deficient mutants and increasing branching of shorter β-1,6-glucan chains in *O*-mannan-deficient mutants.

KRE6-family-members dependent β-1,6-glucan biosynthesis

Genes encoding proteins essential for β-1,6-glucan biosynthesis were first identified in *S. cerevisiae* as mutants resistant to Killer Toxin K1 (*KRE*)[38,66]. In addition, a range of proteins in the endoplasmic reticulum and Golgi (Cwh41, Rot2, Kre5, Kre6, Skn1) are critical for the assembly of this polymer[66]. We analyzed homologs of *CWH41*, *ROT2*, *KRE5* in *C. albicans*. These three calnexin cycle proteins are involved in glucosylation/deglucosylation of *N*-glycan in the ER[67] had a reduced amount of cell wall mannan (**Fig. S2**). Strong cell wall phenotypes were observed for *kre5*Δ/Δ and *rot2*Δ/Δ mutants which exhibited significant increases in the highly branched β-1,3-glucan (x1.8 for *rot2*Δ/Δ, x2.2 for *kre5*Δ/Δ) (**Fig. S4**), chitin (x4.2 for *rot2*Δ/Δ, x5.2 for *kre5*Δ/Δ) and a strong decrease in β-1,6-glucan in the *rot2*Δ/Δ (−68.7%) and *kre5*Δ/Δ mutants (−81.5%) (**Fig. 3g** and **Fig. S2**). In addition, the β-1,6- glucan structure was also altered in both mutants, with a decrease in the average molecular weight (15-16 kDa instead of 43 kDa for the parental strain)

and a reduction in the side chain branching (−54.2% for *rot2*Δ/Δ, and −76.5% for *kre5*Δ/Δ) (**Fig. 3h**, 3i). *KRE1* encodes a plasma membrane protein whose function is not yet clear[68,69]. The *kre1*Δ/Δ *C. albicans* mutant exhibited a reduced molecular weight of β-1,6-glucan chains (20 kDa instead of 43 kDa in the parental strain) (**Fig. 3h**) but was not affected in overall cell wall composition (**Fig. S2**).

In *C. albicans,* the *KRE6* family is composed of four homologs: *KRE6*, *SKN1*, *KRE62* and *SKN2*. These genes encode putative trans-β-glycosylases of the GH-16 family for which no biochemical activity has been described to date[70]. To analyze the function of these four proteins in *C. albicans*, we used a transient CRISPR/Cas9 protocol to build the four single knockout mutants, the double *kre6/skn1*Δ/Δ and the quadruple *kre6*Δ/Δ/*kre62/skn2/skn1*Δ/Δ mutants. Among the single mutants, only *kre6*Δ/Δ exhibited a marked alteration in the cell wall composition (**Fig. S2**), with significantly lower β-1,6-glucan (−46.8%) (**Fig. 3g**) and mannan content (−42.8%) (**Fig. S2**), and higher β-1,3- glucan (x1.9) and chitin (x3.9) content. In addition, the β-1,6-glucan chain length was shorter (−50.5%) and more branched (x1.7) than in the parental strain (**Fig. 3h**, **3i**) and β-1,3-glucan was also hyperbranched (x1.8) (**Fig. S4**). The *kre6/skn1*Δ/Δ mutant phenotype, β-1,6-glucan content and structure were similar to the single *kre6*Δ/Δ mutant (**Fig. 3g**, **3h**, **3i**). The quadruple *kre6/kre62/skn2/skn1*Δ/Δ mutant did not produce any measurable β-1,6-glucan (**Fig. 3g**, **Fig. S2**), which was confirmed by endo-β-1,6-glucanase digestion of the AI and AS fractions (**Fig. S6**). The absence of β-1,6-glucan was associated with an increased chitin content (9.5% vs. 2.5%), and highly branched β-1,3-glucan (48% vs. 20% in the parental strain) (**Fig. S2**, **Fig. S4**). Reintegration of *KRE6* under the control of the *ACT1* promoter restored the production of β-1,6-glucan with a structure similar to that of the parental strain (**Fig. 3g**, **3h**, **3i**).

Therefore, in *C. albicans,* deletion of all *KRE6* homologous genes led to the absence of β-1,6-glucan production and structural alterations of the cell wall, showing that the β-1,6-glucan biosynthetic pathway is critical to the regulation of cell wall homeostasis.

KRE6 regulation of cell wall architecture

The single deletions of *KRE62*, *SKN1* and *SKN2* did not affect the growth of *C. albicans* in SD medium nor drug sensitivity (**Fig. 4**), whereas the deletion of *KRE6* led to a significant increase in doubling time (x2.4, **Fig. 4a, b**). The double *kre6/skn1*Δ/Δ mutant had the same growth rate as *kre6*Δ/Δ (**Fig. 4a, b**). However, deletion of all genes of the *KRE6* homologs family (quadruple *kre6/kre62/skn2/skn1*Δ/Δ mutant) resulted in a stronger growth phenotype with a longer lag phase and a higher doubling time (x3.5 vs. parental strain). Cells of the quadruple *kre6/kre62/skn2/skn1*Δ/Δ mutant were bigger than the parental strain (**Fig. 4d**). In addition, the single *kre6*Δ/Δ and all multiple mutants aggregated in SD liquid medium and had increased sensitivity to the cell wall perturbing agents Congo Red, Calcofluor White, as well as to the chitin synthase inhibitor nikkomycin Z and the *N*-glycosylation inhibitor tunicamycin. No growth of the quadruple mutant was observed in the presence of either of these drugs. Surprisingly, caspofungin enhanced the growth of mutants bearing *KRE6* gene deletion (**Fig. 4c**). Among the single mutants, only *skn1*Δ/Δ was defective for filamentous growth; all multiple mutants also displayed defects in filamentous growth, and the severity of this phenotype was proportional to the number of *KRE6* homologous genes deleted (**Fig. 4d**). The reintegration of *KRE6* under P*ACT1* restored parental strain-like growth kinetics, filamentation and drug resistance (**Fig. 4**) and confirmed the above differences noted in the mutants of *KRE6* and *SKN1*[44].

Cell wall architecture, observed by transmission electron microscopy, showed that single *skn1*Δ/Δ, *kre62*Δ/Δ and *skn2*Δ/Δ mutants presented a bilayered structure with a classical fibrillar outer layer similar to the parental strain (**Fig. 5**). However, the outer layer of the *skn1*Δ/Δ and *skn2*Δ/Δ mutants was thicker than the WT (*skn1*Δ/Δ, 70 ± 12 nm; *skn2*Δ/Δ, 66 ± 12 nm vs. WT, 53 ± 10 nm). The cell wall of the *kre6*Δ/Δ and *kre6/skn1*Δ/Δ mutants had a heterogeneous and thicker inner layer (*kre6*Δ/Δ, 210 ± 89 nm; *kre6/skn1*Δ/Δ, 216 ± 68 nm vs. WT, 108 ± 23 nm) (**Fig. 5**). The external

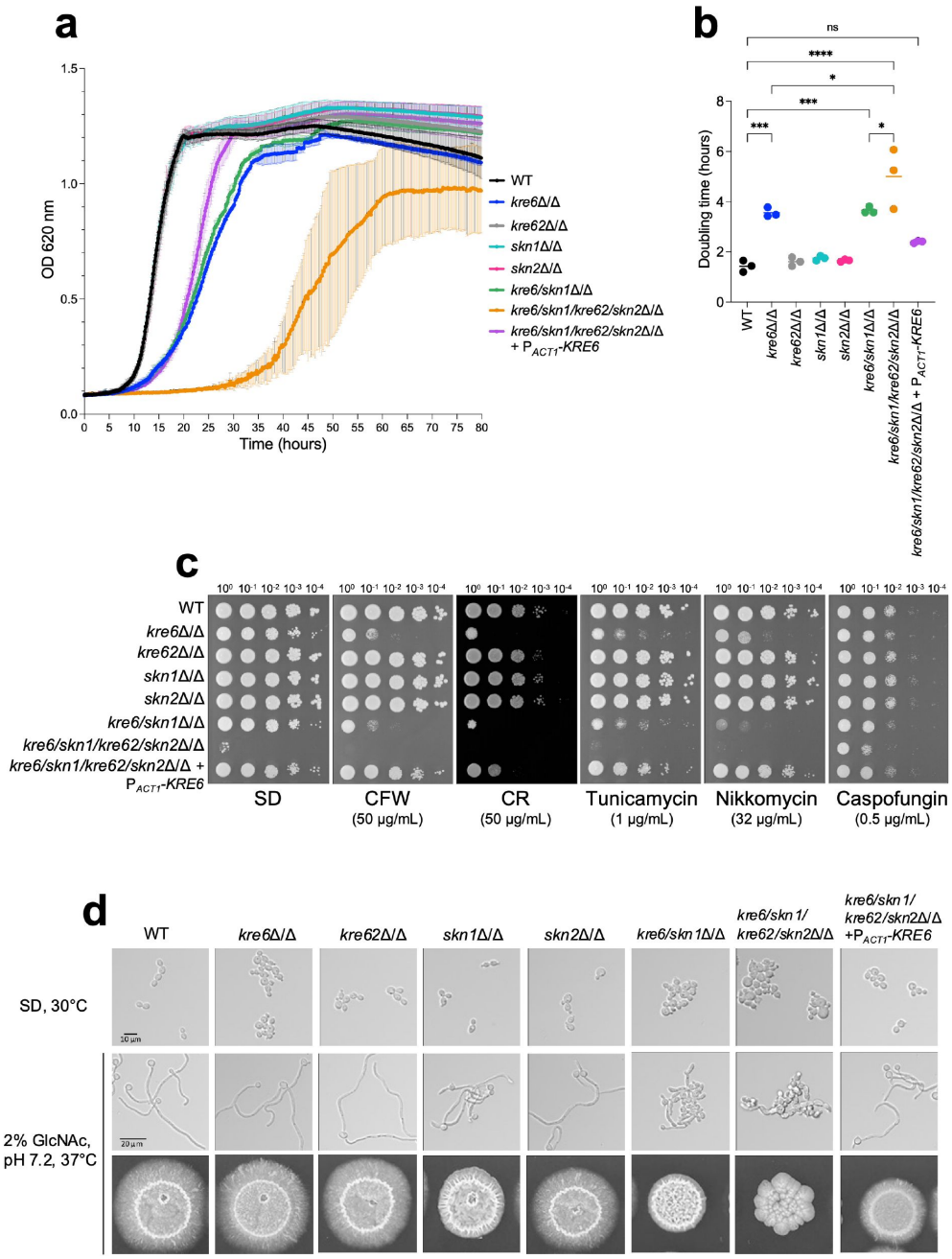

Figure 4

Phenotypic characterization of *KRE6* family mutants: growth kinetics, drug susceptibility and filamentation.

a, Kinetic curve of all strains grown in liquid SD medium, 30°C. Optical density at 620 nm was measured every 10 min during 80 hours by TECAN SUNRISE. Means and standard deviations were calculated from three independent experiments.**b**, Doubling time of each strain was determined from three independent replicates. Statistical analyses were performed with one-way ANOVA with Tukey's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$; ns: non-significant. **c**, Spotting test of 10-fold serial dilution of yeast cells of all strains on SD medium, 30°C, 48h, with cell wall disturbing agents (CR, Congo red; CFW, CalcoFluor White) or drugs (nikkomycin, tunicamycin, caspofungin). These results are representative of three independent experiments. Pictures were taken with a Phenobooth (Singer Instruments).**d,** Filamentation assay of all strains. Top row, growth in liquid SD medium at 30°C; middle panels: growth in liquid YNB medium + 2% GlcNAc, buffered at pH 7.2 at 37°C during 6h. Picture were taken using an Olympus IX 83 microscope, 40x objective. Bottom row, cells were grown on agar YNB + 2% GlcNAc, buffered at pH 7.2, at 37°C for 6 days.

mannoprotein-rich fibrillar layer was absent in the quadruple *kre6/kre62/skn2/skn1*Δ/Δ mutant and the inner layer was thicker and of variable thickness (440 ± 158 nm vs. 108 ± 23 nm). A normal wall architecture was fully restored in the complemented strain.

These results demonstrate that β-1,6-glucan has a pivotal role in determining the architectural arrangement of polysaccharides in the cell wall, which in turn influences growth, drug sensitivity, cell morphology and filamentation.

β-1,6-glucan stimulates human peripheral blood mononuclear cells *in vitro*

The major cell wall polysaccharides of *C. albicans,* β-1,3-glucan, mannan, and chitin function as PAMPs, and their recognition by host PRRs trigger immune responses. Although β-1,6-glucan is one of the major constituent polysaccharide in the cell wall of *C. albicans*, its immunomodulatory potential has been understudied. We extracted β-1,6-glucan from the cell wall of the parental strain and used this to stimulate peripheral blood mononuclear cells (PBMCs) and neutrophils isolated from the whole blood samples of healthy human donors. As controls, PBMCs were stimulated with the AI fraction (composed of β-1,3-glucan, β-1,6-glucan and chitin) as well as with the periodate-oxidized AI fraction (AI-OxP) devoid of β-1,6-glucan. A protein profiler was used to identify cytokines, chemokines and acute phase proteins differentially released by PBMCs and neutrophils stimulated with the three different cell wall fractions (AI, AI-OxP and β-1,6-glucan) (**Fig. S12**, **S13**). Then, differentially stimulated cytokines, chemokines and acute phase proteins were quantified by ELISA. Fractions containing β-1,3-glucan (AI and AI-OxP fractions) induced the secretion of pro- inflammatory cytokines including IL-6, IL-1β and TNF-α, chemokines such as IL-8, MCP-1, MIP-1β and RANTES, and the complement factor C5a (an acute phase protein), but anti-inflammatory cytokine IL-10 was released in a relatively small amount (**Fig. 6a**). However, removal of β-1,6-glucan by periodate oxidation (AI-OxP) led to a significant decrease in the IL-8, IL-6, IL-1β, TNF-α, C5a and IL-10 released, suggesting that their stimulation was in part β-1,6-glucan dependent. Although the response was much lower than that induced by the AI fraction and AI-OxP, β-1,6-glucan activated PBMCs and induced secretion of most cytokines and chemokines (**Fig. 6a**). Both AI and AI-OxP fractions stimulated neutrophils secreted IL-8 at a similar level (**Fig. 6b**) but purified β-1,6-glucan did not, suggesting that β-1,6-glucan and β-1,3-glucan stimulate innate immune cells in distinct ways. Because the structure of cell wall β-1,6-glucan is dependent on stress as well as environmental conditions of growth, we investigated the structure-function immune response of β-1,6-glucan from *C. albicans*. Shorter β-1,6-glucan chains (19 kDa) were purified from the cell wall of *C. albicans* cell wall grown with lactate as carbon source and larger β-1,6-glucan chains (70 kDa) were purified from cell wall produced in the presence of caspofungin (**Fig. 2**). Both fractions stimulated PBMCs and led to cytokine/chemokine secretion at a level similar to the control β-1,6-glucan, suggesting that β-1,6-glucan size was not a significant factor influencing the stimulation of PBMCs (**Fig. S5**).

Discussion

Cell wall β-1,6-glucan is a conserved constituent in most of the major human fungal pathogens including *Candida*, *Cryptococcus*, *Pneumocystis*, *Histoplasma* and *Malassezia* species [47,71,72]. The cell wall of fungal pathogens is the target of several classes of antifungal drugs and is critical for immune recognition and activation, therefore has been the subject of numerous investigations[29,47]. However, this polysaccharide has been neglected, perhaps because its analysis requires bespoke methodologies. Here, we established new biochemical tools for quantitative and comparative analysis of cell wall β-1,6-glucan dynamics and structure in *C. albicans* and used them to demonstrate that this polysaccharide is instrumental in determining the gross architecture of the fungal wall and its immune reactivity.

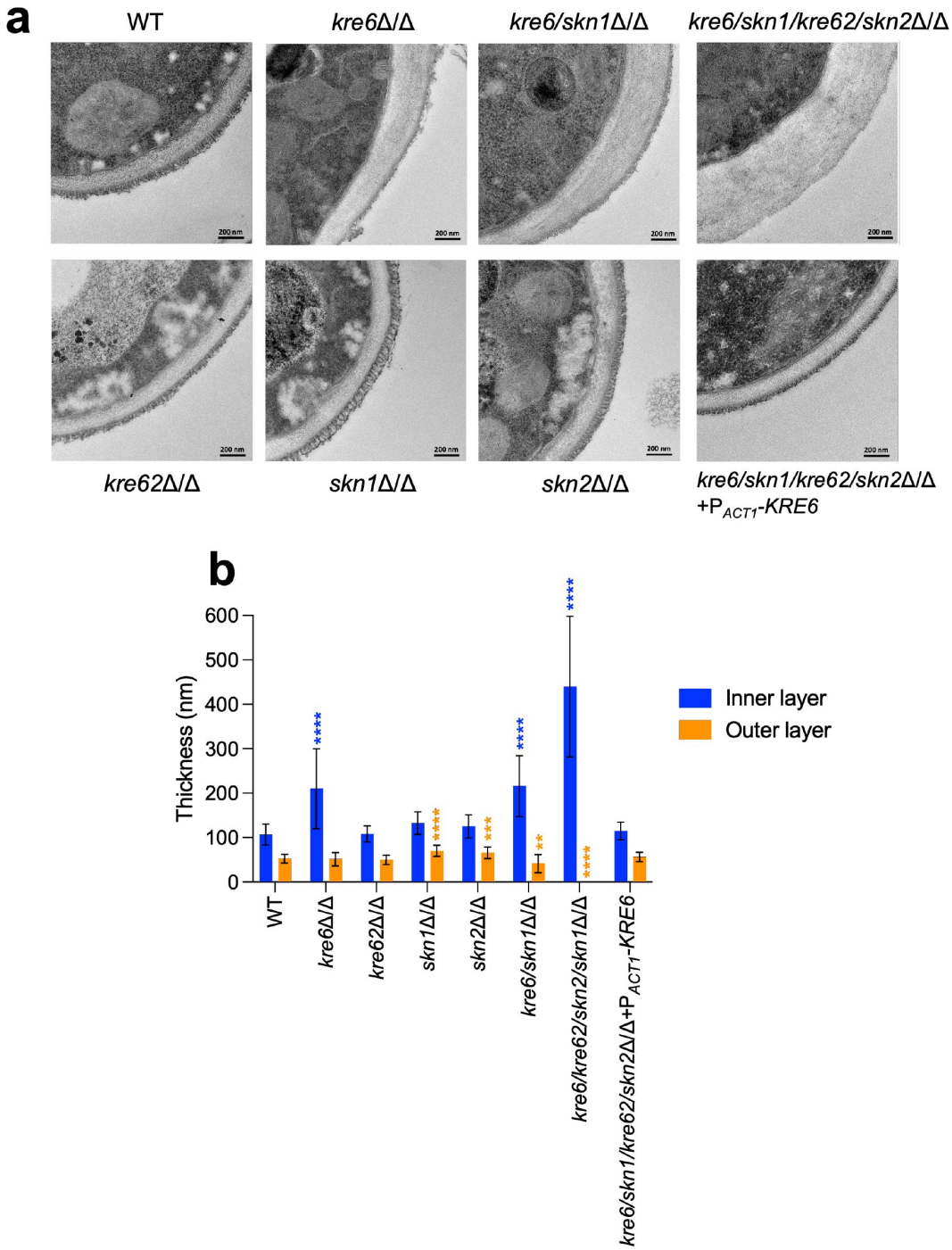

Figure 5

Cell wall electron microscopy observations of *KRE6* simple and multiple mutants.

a, Representative transmission electron microscopy images of the cell wall of each strains. After culture, cells were fixed and high-pressure frozen and freeze substituted with Spurr resin. Sections were cut and stained and then pictures were taken by a Tecnai Spirit 120Kv TEM microscope. Scale bar= 200 nm.

b, Measurement of the inner and outer cell wall layers of the mutants. Means and standard deviations are represented. 37-40 measurements were performed randomly on 7-13 cells.

Statistical analyses were performed with one-way ANOVA with Tukey's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$.

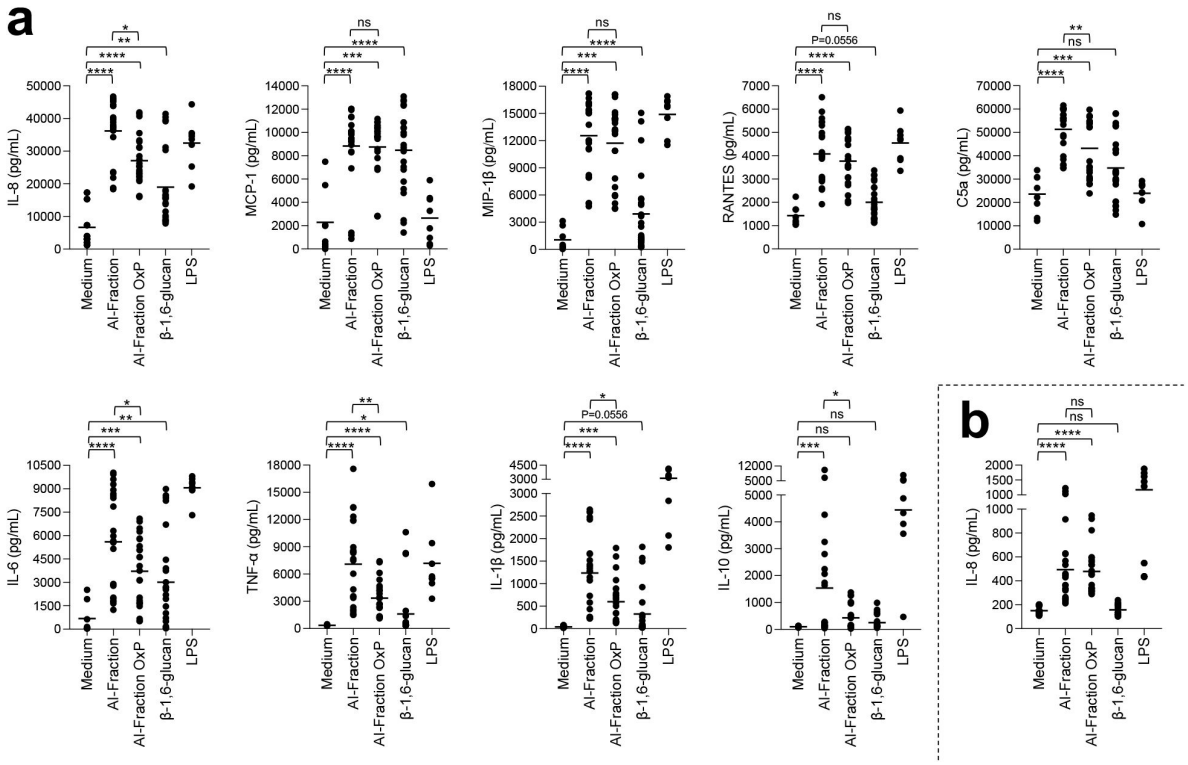

Figure 6

Stimulation of PBMCs and neutrophils *in vitro* by cell wall fractions and purified β- 1,6-glucans from *C. albicans.*

Cytokines, chemokines or acute phase proteins (IL-8, MCP-1, IL-6, MIP-1β, IL-1β, TNF-α, RANTES, C5a, IL-10) concentrations in culture supernatants of PBMCs (**a**) and neutrophils (**b**) stimulated by cell wall fractions of *C. albicans* (AI-Fraction, AI-Fraction OxP and β-1,6-glucan) at 25 µg/mL or LPS (positive control, 0.1 µg/mL). PBMC cells and neutrophils were isolated from healthy human donors (n=8). Three independent batches of each fractions were used. Means are represented and data were analyzed using nonparametric Friedman test with Dunn's multiple comparisons: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$; ns: non-significant.

In SD medium at 37°C, β-1,6-glucan represents 23% of the total cell wall, with an average size of 58 kDa (**Fig. 1**). From our analytical data on *C. albicans* cell wall, we proposed an updated model underlining the true role of β-1,6-glucan as a major cell wall component (**Fig. 7a**). NMR has demonstrated that the structure of β-1,6-glucan in the yeast cell wall is similar between species[33,71], but there are variations in β-1,6-glucan content, chain length and branching. *Malassezia restricta* contains long β-1,6-glucan chains, averaging 80 kDa, whilst in *S. cerevisiae* and *C. albicans* they are moderate in size (30-60 kDa). We showed here that modulating environmental conditions induce significant changes in both cell wall structure and content. As described previously, lactate used as the sole carbon source led to a thinner inner cell wall layer and greater exposure of β-1,3-glucan[51,73]. We observe here that lactate medium also led to a marked reduction in cell wall β-1,6-glucan content and chain length (**Fig. 2**, **Fig. 7b**). Our data showed that several factors related to host niches (morphology, temperature, salinity, pH and presence of organic acids) and the presence of antifungals had a significant impact on the structure, size and branching of β-1,6-glucan (**Fig. 7b**). This dynamic nature of the β-1,6-glucan component of the wall (**Fig. 2**, **Fig. S1** and **Fig. S2**) is as important that the regulation and structural diversity of chitin, mannan and β-1,3-glucan[64].

The dynamic regulation of mannan biosynthesis leading to structural changes in the outer mannoprotein layer of *C. albicans* is also under-investigated. *C. albicans* evolves in different environments as a commensal or pathogen[4], mainly in the gut, oral cavity, genital tract and blood. How fungal cell walls adapt to changing environments was recently been listed as one of the top five unanswered question in fungal cell surface research[74]. Our data demonstrate that in response to several environmental factors β-1,6-glucan and mannan productions are coupled. The *in vivo* importance of this novel dynamic process remains to be addressed but it could be critical in the articulation of host inflammatory responses[75,76].

Immunolabelling with an anti-β-1,6-glucan serum demonstrated that this polymer is exposed at the cell surface and is therefore accessible to receptors that stimulate human immune cells (**Fig. S11** and **Fig. 6**). Similarly in *Pneumocystis carinii*, β-1,6-glucans are exposed to the cell surface and stimulate mouse macrophages leading to the release of TNF-α[72]. Our immunological data indicate that β-1,6-glucan is a *bona fide* fungal cell wall PAMP that activates the human immune system (**Fig. 7d**). Beads coated with pustulan (an insoluble linear form of β-1,6-glucan) activate neutrophils and induce massive ROS production and phagocytosis in a dectin-1-independent manner[77,78]. We observed that the absence of human serum in the culture medium led to a strong reduction of β-1,6- glucan induced cytokine secretion from PBMCs and that β-1,6-glucan bind to C3b (**Fig. S14**). These data are in agreement with the binding of pustulan to C3b/C3d, as described previously[77,78]. This allow us to assume that the opsonization by complement component C3 could partially mediated the recognition of β-1,6-glucan by immune cells, but needs to be validated experimentally. We observed that the size of soluble β-1,6-glucan did not alter immune stimulation *in vitro* (**Fig. S5**) whereas, despite its ability to bind soluble and particulate β-1,3-glucan polymers, Dectin-1 signaling is only activated by particulate β-1,3-glucans[79]. The relevance of fungal β-1,6-glucan to the global host immune response and mechanism of recognition remain to be investigated.

In *C. albicans*, β-1,3-glucan exposure is regulated by external signals such as hypoxia, carbon source or iron deprivation via the up-regulation of β-1,3-glucanase activities that lead to β-1,3-glucan masking and immune evasion[75,80,81]. Regulation of the mechanisms determining β-1,6-glucan exposure and the expression of secreted glucanases remains also unknown in *C. albicans*. The absence of β-1,6-glucan in the AI fraction led to a reduced inflammatory response (**Fig. 6**), suggesting that an endo-β-1,3-glucanase, such as Eng1, may enable the release of cross-linked β- 1,6-glucan from the cell surface, which could attenuate the host response by pruning β-1,6-glucans that are covalently bound to β-1,3-glucans from the surface [81].

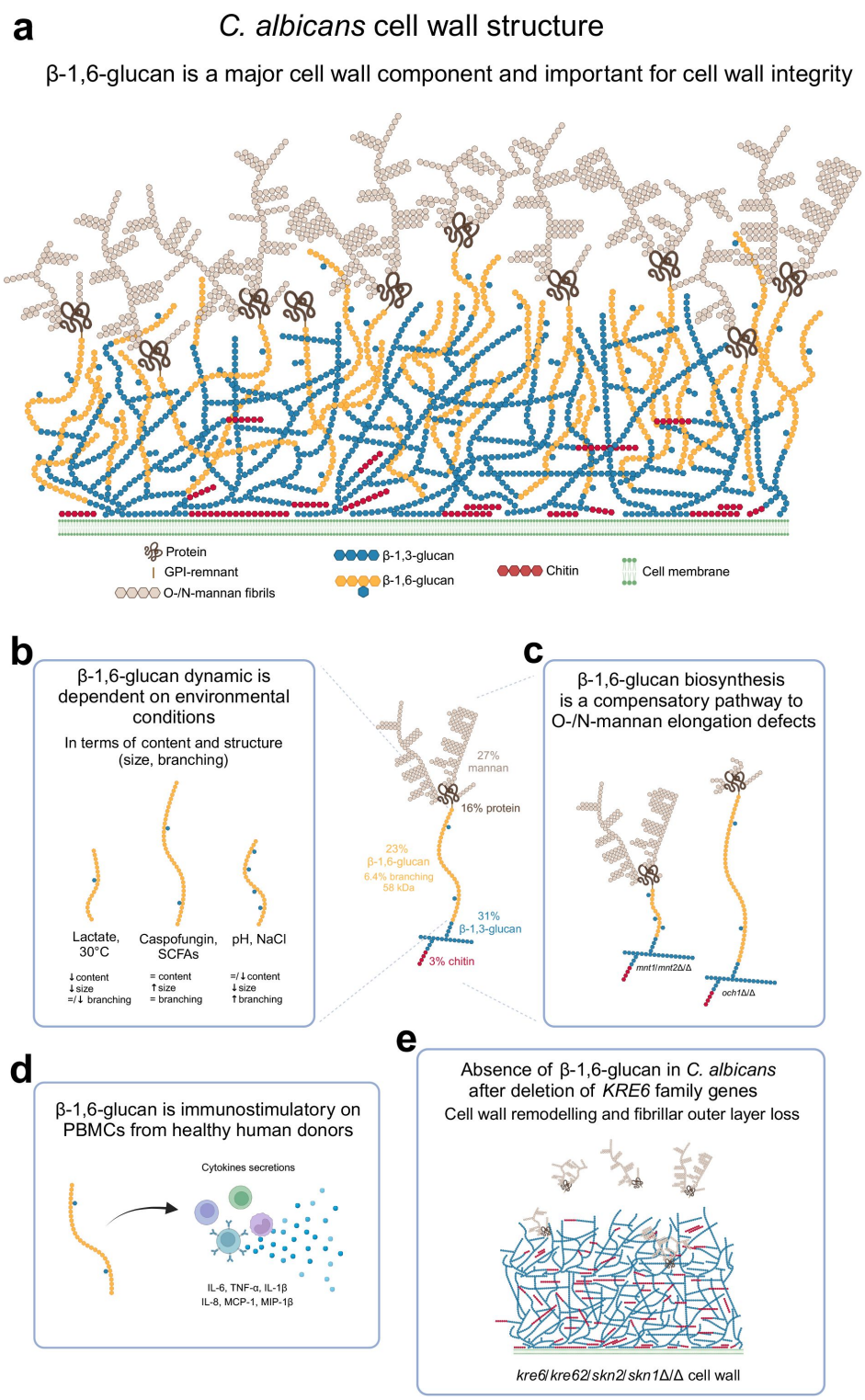

Figure 7

β-1,6-glucan in *C. albicans* is a major and dynamic cell wall polymer.

a, Scheme of the cell wall of *C. albicans*. The proportion of each cell wall polymer was representative the results obtained on *C. albicans* SC5314 grown in liquid SD medium at 37°C. **b,** Scheme representing the dynamic of β-1,6-glucan under different environmental factors. **c**, β-1,6-glucan is a compensatory pathway for mannan elongation defect. **d**, β-1,6-glucan is a PAMP. **e**, Scheme of the cell wall of *KRE6* family deficient mutant.

Cell wall organization in *C. albicans* depends on the cross-linking of all polymers that function as a dynamic flexible network [29,47]. Their interconnectedness means that alteration in the synthesis of one polymer can lead to a compensatory change and cell wall remodelling. Our data provides one new example of this phenomenon – namely decrease in cell wall chitin content results in an increase of both β-1,3-glucans and β-1,6-glucans in the AS fraction of the wall. Chitin exists in the yeast cell wall in three forms: free (~40%), and crosslinked to β-1,3-glucan (40-45%) or β-1,6-glucan (15-20%) and crosslinked β-glucan-chitin forms the core fibrillar cell wall structure resistant to alkali- solubilization[29,82–84]. Therefore, a decrease in the chitin content might reduce the formation of fibrillar structure, thereby releasing the β-glucan contents into the AS-fraction. On other hand, in the *FKS1* mutant or upon caspofungin treatment, cell wall chitin and β-glucan-chitin contents were increased, which could be attributed to an upregulated *CRH1* expression due decreased β-1,3-glucan synthesis as in *FKS1* mutant[85] or due to caspofungin treatment. *CRH* family members are involved in the β- glucan-chitin crosslinking[86]. Interestingly, defects in the expression of *CHS1* that encodes an essential chitin synthase required to construct the primary septum, was also compensated by an increased content of cell wall β-1,6-glucan but not of β-1,3-glucan (**Fig. 3**). This suggests that Chs1 has other functions beyond septum construction, that had previously been suggested by loss of cell integrity and swelling of cells upon *CHS1* repression[87]. In *S. cerevisiae*, deletion of *GAS1*, the ortholog of *PHR1* and *PHR2*, led to a strong decrease of cross-linked β-1,6-glucan to β-1,3-glucan- chitin cell wall core[62]. Deletions of *PHR1* and *PHR2*, holding a β-1,3-glucan remodelling activity, led to an increased chitin content (**Fig. S2**) and to a reduction of β-1,3-glucan branching (**Fig. S4**). However, a decrease in β-1,6-glucan content and β-1,6-glucan size was only observed in the *PHR2* mutant (**Fig. 3**). As *PHR1* and *PHR2* genes are strongly regulated by external pH, the compensatory differences we described may be explained by pH dependent regulation of β-1,6-glucan synthesis (**Fig. 3**).

In *C. albicans*, the mannan moieties of glycoproteins are covalently cross-linked to the β-glucan-chitin core. Mutants defective in *N*-mannan elongation led to a marked increase in β-1,6-glucan content and size (**Fig. 3**). In yeast, Och1 and Mnn9 are α-1,6-mannosyltransferases that are involved in initial steps of α-1,6-mannan polymerization in the Golgi. Members of the Mnn2 α-1,2 mannosyltransferases are required for the addition of the side chains of the α-1,6-mannan backbone[23]. Mutants in these genes led to defects in the outer fibrillar layer[27,88] and increased β-1,3- glucan exposure[23,89]. The quantification of β-1,6-glucan content in these *N*-mannan deficient mutants had not been described previously in *C. albicans*[23,88] and underline the new finding that the regulation of mannan, β-glucans and chitin synthesis is coordinated and coupled. Screening with K1 toxin killer revealed a hypersensitivity of the Sc*mnn9* mutant and an increase in β-1,6-glucan cell wall content[90] reinforcing the hypothesis that β-1,6-glucans play a compensatory role when *N*-mannosylation is compromised (**Fig. 7c**). Thus, a defect in the fibrillar outer mannan layer led to an increase in the inner cell wall chitin and β-1,6-glucan contents (**Fig. S2**), suggesting their synthesis was regulated by a rescue mechanism associated with the reprogramming of specific transcriptional responses via the cell wall integrity (CWI) pathway[91]. Defects in *O*-mannosylation also affected β-1,6-glucan synthesis leading to shortened highly branched β-1,6-glucan chains, without significant changes in overall cell wall composition, suggesting a specific inhibition of the elongation of β-1,6-glucan chain and showing that size and branching of β-1,6-glucan can be regulated independently (**Fig. 7c**).

Fine details of the biosynthetic pathway of yeast β-1,6-glucans are not clear. In *S. cerevisiae*, deletion of *KRE5* and *ROT2* led to a reduction in cell wall β-1,6-glucans[66]. In *C. albicans*, this reduction is characterized by the presence of short seldom-branched β-1,6-glucan chains (**Fig. 3**). Although no difference was observed in the *CWH41* mutant, our data imply that canonical *N*-glycan processing in the ER is required for complete β-1,6-glucan synthesis[90], and that Kre5p is not the β- 1,6-glucan polymerase[35]. Furthermore, our results confirm that the β-1,6-glucan biosynthetic pathway is similar in these phylogenetically distant yeasts, and that the *N*-glycosylation pathway is critical for β-1,6-glucan synthesis. Defects in early *N*-glycan synthesis at the ER level led to concomitant defects in β-1,6-glucan synthesis while defects in *N*-mannan

elongation led to increased β-1,6-glucan synthesis. However, as Kre5, Rot2 and Cwh41 are part of the calnexin cycle involved in the control of N-glycoprotein folding in the ER, early N-glycan synthesis may have an indirect effect in ensuring the functionality of the enzymes rather than the synthesis of N-glycan moiety required for β-1,6-glucan synthesis. Our hypothesis is that this biosynthesis begins intracellularly. According to *in vitro* systems in *S. cerevisiae*, the initial step is the polymerization of a linear chain by a β-glucosyltransferase using UDP-glucose as the sugar donor[33,92] and probably an as yet unknown acceptor (**Fig. S15**). The second step would be the addition of glucoside and laminaribioside as a side chain. Finally, after release into the cell wall, β-1,6-glucan will be cross- linked to the other cell wall polymers. Among the *KRE* genes, only *KRE6* may be directly involved in β-1,6-glucan synthesis. Kre6 and its homologs contain a β-glycosyl-hydrolase domain of the GH-16 family, which includes a large number of fungal enzymes such as Crh1 homologs that are involved in the cross-linking of chitin and β-1,3-glucan[86]. In *C. albicans*, the quadruple deletion of *KRE6*, *SKN1*, *SKN2* and *KRE62*, led to the complete absence of β-1,6-glucan in the cell wall (**Fig. 3**, **Fig. 7e**) but also in culture supernatants and in intracellular extracts (not shown), demonstrating a stop of β-1,6-glucan synthesis rather than miss-localization. The absence of β-1,6-glucan in the quadruple mutant led to global cell wall remodeling and the absence of the outer fibrillar *N*-mannan layer (**Fig. 5** and **Fig. 7e**). Kre6 had the dominant role and its absence led to a hypersensitivity to cell wall- targeting antifungal drugs[34,44]. One exception was observed for caspofungin which enhanced the growth of all *KRE6* deleted mutants (**Fig. 4c**). Growth above the MIC (paradoxical growth) has been described at high caspofungin concentrations and is associated with high chitin content[43,93] as observed in these mutants. Our data show that Kre6 is required for production of normal amounts of β-1,6-glucan and that the remaining β-1,6-glucans present in *kre6*Δ/Δ was composed of short and highly branched chains. This suggests that the Kre6 family is required for the polymerization and/or elongation of β-1,6-glucan, but not glucan branching.

In *S. cerevisiae*, only two *KRE6* family members have been identified, Kre6 and Skn1, and their absence led to strong growth defects and hypersensitivity to cell wall perturbing agents –similar to what we observed here in *C. albicans* [94]. In *C. neoformans*, among the six *KRE6* homologs, *KRE6* and *SKN1* are required for the production of cell wall β-1,6-glucan[95] and although the *KRE6* family is essential for β-1,6-glucan synthesis, its biochemical function remains unknown. Kre6 homologs are type II membrane proteins with a GH-16 domain at the luminal C-terminus and an unfolded cytosolic *N*-terminal tail[96]. Yeast Kre6 and Skn1 proteins have moderate similarity with diatom TGS (1,6-β-transglycosylase) proteins[97,98] and can synthesize glucan from UDP-Glc[99]. Kre6 homologs share structural similarities with β-glucanases and β-transglycosylases, arguing for a possible cell wall remodeling function. In *S. cerevisiae*, Kre6p has been localized in the endoplasmic reticulum, the plasma membrane and secretory vesicle-like compartments[66,96,100], and the cytosol tail was shown to be essential for the correct cellular localization and β-1,6-glucan synthesis[96]. Surprisingly, in *C. albicans* the cytosol tail is not essential[44], suggesting some functional differences of this protein in these yeasts. In *C. albicans*, the double *KRE6/SKN1* mutant is avirulent in a mice model of candidiasis[44], showing that the β-1,6-glucan biosynthetic pathway is a potential virulence target. However, the drugs Jervine (steroidal alkaloid) and a pyridobenzimidazole derivate (D75-4590), that may target Kre6 and inhibit *S. cerevisiae* growth[101,102], had no effect on *C. albicans*[101,102], presumably representing species-specific differences in drug profiles. Kre6 family members are present in all fungal pathogens producing cell wall β-1,6-glucans and further investigations are required to understand the enzyme activities and the diversity of structures related to the potential drug-targeting of these proteins.

Overall, this work highlights the central role that β-1,6-glucan plays in orchestrating cell wall synthesis during growth and in response to stress and sets the focus for further work that can exploit this central role in mitigating fungal infections.

Methods

Ethics statement

Human blood samples were taken from healthy donors from Etablissement Français du Sang Trinité (Paris, France) with written and informed consent, as per the guidelines provided by the institutional ethics committee, Institut Pasteur (convention 12/EFS/023).

Strains and growth conditions

The *Candida albicans* strains used in this study are listed in **Table S1**. The reference strain SC5314, used for all stress conditions, was grown overnight in 25 mL flasks, at 37°C, 180 rpm, in SD medium (2% glucose, 0.67% yeast nitrogen base with amino acids (YNB) (BD), pH 5.4). Overnight cultures were diluted to an OD600= 0.001, in 50 mL fresh SD medium, incubated at 37°C, 180 rpm, and then harvested either when reaching mid-exponential phase (OD600 ~ 3-5) or at stationary phase (OD600 ~ 11-12 after 30h). The following growth conditions were selected: hypha induction (SD containing 2% GlcNAc instead of 2% glucose, pH 7.2, no yeast-form was observed in this medium), temperature (30°C), osmolarity (1M NaCl), carbon source (SD containing 2% lactate (ChemCruz) instead of 2% glucose), oxygen limitation (hypoxia: 1% O2, 5% CO2, anaerobic chamber (Baker Ruskinn, I-CONIC, Bugbox M)) and oxidative stress (H2O2 1.5 mM, Prolabo). For studies of the effect of medium pH, SD medium was buffered with 100 mM MES (Sigma) at pH 4 or 5.4 or with MOPS (Sigma) at pH 7.5. For experiments on the effects of short-chain fatty acids (SCFAs), SD medium was supplemented with 100 mM MES and 50 mM acetate (Sigma) or 20 mM propionate (Sigma) or 15 mM butyrate (Sigma) and buffered to pH 5.4. Drugs were tested at sub-lethal concentrations: Calcofluor White (CFW, 400 µg/mL, Sigma-Aldrich), Congo Red (CR, 50 µg/mL, Sigma), tunicamycin (0.125 µg/mL, Sigma), nikkomycin (16 µg/mL, Sigma) and caspofungin (0.015 µg/mL, Sigma). Biofilm production was induced in SD medium at 37°C for 48h in 6 well plates as previously described with some modifications[103]. Overnight cultures were diluted at OD600=0.2 in 6 well polystyrene plates (TPP) in 2 mL of minimal medium (SD: 2% glucose, 0.67% yeast nitrogen base without amino acids (BD), pH 5.4) supplemented by arginine 0.1 g/L, histidine 0.1g/L, uridine 0.02 g/L and methionine 0.2 g/L. The plates were incubated at 37°C, 60 min at 110 rpm for initial cell adhesion. Then, the plates were washed with 2 mL of fresh medium, and 4 mL of fresh medium was added. Plates were then sealed with Breathseal sealing membranes (Greiner Bio-one) and incubated at 37°C, 110 rpm. After 48 h, supernatant culture was aspirated, the wells were gently washed with PBS and biofilm was collected in PBS.

Cell wall mutant strains were grown at 30°C, 180 rpm, in SD medium supplemented with arginine (100 µg/mL), uridine (100 µg/mL) and histidine (20 µg/mL). The *phr1*Δ/Δ mutant was grown in SD buffered medium at pH 7.5 (with 100 mM MOPS), and the *phr2*Δ/Δ mutant at pH 5.5 (with 100 mM MES). The conditional mutant P*MRP1-CHS1/chs1*Δ was first precultured in YNB with 2% maltose as a carbon source to allow the *MRP1* promoter expression and then it was grown, as all other mutants, in SD (carbon source glucose), under which condition the promoter is repressed.

Cell wall fractionation

After growth culture, the fungal biomass was collected by centrifugation (5 min, 3300 g), washed and disrupted in 200 mM Tris-HCl pH 8 using a cell disruptor (FastPrep, MPbio, 6 cycles, 6 m/s, 60 sec) with 0.3 mm glass beads (Braun). The cell wall was collected by centrifugation (5 min, 3300 g), washed twice with 200 mM Tris-HCl, pH 8, then twice with milliQ water and freeze-dried. The wall polymers were then fractionated as previously described[104]. Briefly, non-covalently bound proteins were extracted by the following treatment: dried cell wall was treated twice in 50 mM Tris-HCl, 50 mM EDTA, pH 7.5, 2% SDS, 40 mM β-mercaptoethanol in boiling water for 10 min (10-20 mg of cell wall/mL). After centrifugation (10 min, 3300 g), three volumes of ethanol were added

to the supernatant and kept overnight at +4°C. The precipitate (SDS-β-ME fraction) was collected by centrifugation (10 min, 3300 g) and dialyzed against water before freeze-drying. Other cell wall pellet was washed three times with 150 mM NaCl and extracted twice with a 1 M NaOH containing 1 mg/mL BH4Na (Sigma), at 10-20 mg of cell wall/mL at 65°C for 1 h. After centrifugation (10 min, 3300 g), the alkali-soluble extract (AS fraction, supernatant) was neutralized with acetic acid, extensively dialyzed against water and freeze-dried. The alkali-insoluble fraction (AI fraction, pellet) was washed three times with distilled water and freeze-dried. Of note, the cell disruption was a key step to remove glycogen from cell wall extracts (**Fig. S7**).

Cell wall polymers quantification

Cell wall polymers of the three cell wall fractions (SDS-β-ME, AI and AS fractions) were quantified as previously described[104]. Briefly, total neutral sugars were quantified by the phenol-sulfuric acid colorimetric assay using glucose as a standard[105]. Proteins were quantified by colorimetry using the Pierce^{TM} BCA Protein Assay Kit (ThermoScientific) according to the manufacturer's instructions. Monosaccharides, glucose and mannose, were identified and quantified by gas liquid chromatography (Perichrom) as their alditol acetates obtained after hydrolysis (4 N trifluoroacetic acid (TFA, Sigma), 100 °C, 4 h)[106]. Glucosamine was quantified by high performance anion exchange chromatography (HPAEC, Dionex, ICS-3000) on a CarboPAC-PA1 column (ThermoScientific, 250 mm x 4.6 mm) after acid hydrolysis (6 M HCl, 6 h, 100 °C) using glucosamine as a standard. Samples were eluted at a flow rate of 1 mL/min with 18 mM NaOH for 15 min, then with a linear gradient to 300 mM sodium acetate (AcONa) in 100 mM NaOH for 20 min and finally under isocratic conditions with 300 mM AcONa in 100 mM NaOH for 5 min. The column was equilibrated for 20 min with the initial buffer before injection. Samples were detected on a pulsed electrochemical detector.

Quantification of β-1,6-glucan in alkali-insoluble (AI) and alkali-soluble (AS) fractions

To quantify β-1,6-glucan in the AI fraction, a colorimetric assay was used. Oxidation with periodate allows specific degradation of vicinal carbons bearing hydroxyl groups. In the AI fraction, only β-1,6- glucans were sensitive to this treatment, leading to the formation of aldehyde functions, which were quantified in the presence of 4-hydroxybenzhydrazide (PAHBAH) (Sigma) (**Fig. S8**). Oxidation was carried out at 50°C for 6 h on 100 µL of AI fraction (0.5 mg/mL) with 100 µL of 25 mM *m*-IO4Na (Sigma-Aldrich). Excess of reagent was quenched with 100 µL sodium sulfite 200 mM (Sigma). Then, 100 µL of reaction mixture were added to 900 µL of PAHBAH reagent (0.63 g sodium sulfite, 5 mL NaOH 5 M, 5 mL sodium citrate 0.5 M, 5 mL NaCl2 0.2 M qsp 100 mL milliQ water, 1 g PAHBAH) and placed in a boiling water bath for 10 min. The quantification of produced aldehyde was measured by the absorbance at 405 nm. Pustulan (linear β-1,6-glucan, Elicityl OligoTech) was used as a standard. The β-1,3-glucan content of the AI fraction was calculated as the total glucose content after subtraction of the amount of β-1,6-glucan.

The presence of mannan in the AS fraction prevents to use the colorimetric assay of β-1,6-glucan after periodate oxidation. β-1,3-glucan and β-1,6-glucan of the AS fraction were therefore quantified by enzyme digestion. The AS fraction was digested with the endo-β-1,3-glucanase, LamA (from *Thermotoga neapolitana* expressed in *E. coli*)[107] or with an endo-β-1,6-glucanase (from *Schizosaccharomyces pombe* expressed in *Pichia pastoris*)[108]. Digestions were carried out at 37°C during 24 h by treating 0.2 mg of AS fraction with 20 µL of LamA (specific activity: 10 µmol eq/min/µL) or 60 µL of the endo-β-1,6-glucanase (specific activity: $3.03x10^{-6}$ µmol eq/min/µL), in 50 mM NaOAc, pH 6, in a final volume of 200 µL. Both enzymatic treatments released more than 95% of glucan. Products were quantified by the amount of released reducing sugars using the PAHBAH reagent[109] and identified by HPAEC on a CarboPAC-PA1 column (ThermoScientific). Soluble and purified β- 1,6-glucans from *C. albicans* and a commercial source of laminarin (Sigma) were used as standards. Products from enzymatic digestion were eluted under the following gradient: isocratic step of 98% of eluent A (50 mM NaOH) and 2% of eluent B (500 mM AcONa in 50 mM

NaOH) for 2 min, 2–15 min of a linear gradient of AcONa in 50 mM NaOH (2% B to 20% B), 15–20 min of a linear gradient (20% B to 43% B), 20–22 min of a linear gradient (43% B to 100% B), 22–25 min under isocratic conditions with 100% B. The column was equilibrated for 20 min with initial buffer before injection.

Structural characterization of β-1,6-glucan

β-1,6-glucan are branched by β-1,3 linked glucose or laminaribiose on the main chain[32,33]. Branching was estimated by enzymatic digestion by an endo-β-1,6-glucanase as previously described[33]. A 0.1 mg sample of the AI fraction was digested for 48 h at 37°C in a final volume of 100 µL with 30 µL of endo-β-1,6-glucanase in 50 mM sodium acetate, pH 6, 5 mM NaN3. Degradation products were analysed by HPAEC on a CarboPAC-PA1 column as described above. Branching was estimated by the ratio of branched oligosaccharides on total degradation products, to which a coefficient of correction was applied. This coefficient (43.1) was calculated from a branching rate of 6.9% of purified β-1,6-glucan established by NMR (see below and **table S2**). Branching of β-1,3-glucan was determined in the same way after digestion of the AI fraction with LamA.

The size of the β-1,6-glucan chain was estimated by gel filtration chromatography. First, β-1,6-glucans were released by incubation at 37°C for 48 h of the AI fraction (0.5 mg) with LamA (100 µL) in 70 mM sodium acetate, pH 6, 5 mM NaN3, in a final volume of 700 µL. After 3 min centrifugation at 12000 g, the supernatant was concentrated under vacuum (SpeedVac concentrator). Then, the sample was submitted to a gel filtration on a Superdex^{TM} 200 column (300 × 10 mm, Cytiva), eluted with ammonium acetate 150 mM, pH 4 at a flow rate of 0.2 mL/min. Samples were detected by a refractive index detector (Iota-2, Precision Instruments). The column was calibrated with dextran molecular weight standards (6, 10, 40, 70 and 500 kDa; GE Healthcare).

Nuclear magnetic resonance (NMR)

NMR experiments were recorded at 318.15 °K using a Bruker (Billerica, USA) 800 MHz Avance NEO spectrometer with an 18.8 Tesla magnetic field equipped with a cryogenically-cooled triple resonance (^{1}H,^{13}C,^{15}N) TCI probe. Spectra were recorded using TopSpin 4.2 (Bruker) and analyzed with CCPNMR Analysis 2.5.2[110]. ^{1}H and ^{13}C chemical shifts were referenced to external DSS (2,2-dimethyl-2-silapentane-5-sulfonate, sodium salt). After three cycles of exchange (dissolution/lyophilization) against D2O, 5 mg of purified polysaccharide were dissolved in 550 µL of D2O (99.99% 2H, Eurisotop, Saclay, France) and placed in a 5 mm tube (Norell HT). Resonance assignment, glycosidic bonds identification and J coupling determination were achieved following standard procedures from homonuclear ^{1}H 1D, 2D COSY[111], NOESY[112] (150 ms mixing time) and natural abundance heteronuclear ^{1}H-^{13}C experiments: edited HSQC[113,114], CLIP-HSQC[115], H2BC[116], HMBC[117] and HSQC-TOCSY[114] (100 ms mixing time). The anomeric configuration of monosaccharide residues was established from the corresponding chemical shifts and ^{1}JH1-C1 coupling constants obtained from CLIP-HSQC spectra. Glycosidic bonds were identified using HMBC experiments and confirmed with NOESY data. ^{3}JH1-H2 coupling constants were measured from ^{1}H 1D spectra. The relative amount of monosaccharide residues was estimated from the integrals on ^{1}H 1D spectra obtained at 333.15 °K using a total recovery delay of 12 s.

Transmission electron microscopy

Cells were grown as described above and volumes of culture media equivalent to a quantity of cells equivalent to OD600nm=1 were collected and centrifuged 5 min at 3300 g. Cells were chemically fixed at room temperature in the dark for 1 h with 2.5% glutaraldehyde (Sigma) in fresh culture media (10 mL), washed in 1X PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2, pH 7.3) and centrifugated at low speed. The pellets were then resuspended in a droplet of 1X PHEM buffer and taken up into cellulose capillary tubes. For each strain, the

capillaries were cut into 3 mm pieces, placed in 6 mm type A specimen carrier (side 0.2 mm) filled with hexadecen, covered by 6 mm type B carrier (side flat) and frozen with a high-pressure freezing machine (EM ICE, Leica microsystems) as previously described[118]. The samples were then freeze-substituted in a mix of 1% OsO4, 5% H2O in pure acetone for 24 h at −90°C, 12 h at 5°C/h at −30°C, 12 h at −30°C, 3 h at 10°C/h until 0°C and 1 h at 0°C. All samples were washed in pure acetone, and gradually infiltrated under agitation in low viscosity Spurr resin/acetone mix from 30% to 100%, as previously described[119]. Samples were then embedded in pure Spurr resin (EMS), followed by polymerization for 48 h at 60°C. Ultrathin sections (70 nm) were cut with a Leica Ultracut S microtome, and stained with 4% uranyl acetate and then 3% lead citrate. Transmission electron microscopy (TEM) images were captured with a Tecnai Spirit 120 kV TEM equipped with a bottom-mounted Eagle 4kx4k camera (FEI, USA). For statistical analysis, internal and external cell wall thickness have been measured (between 37-40 measurements, on 7-13 cells per condition) using TEM Imaging and Analysis software (TIA, FEI, USA).

Immunolabeling

Rabbit polyclonal antibodies were produced by ProteoGenix (Schiltigheim, France) using pustulan-conjugated BSA as the antigen. After 7-8 weeks of immunization, the resulting serum was used without antibodies purification. The specificity of serum was tested on pustulan, curdlan and cell wall AI fractions from *Aspergillus fumigatus* (which did not contain β-1,6-glucan) and *C. albicans* by ELISA (not shown). For immunolabelling of β-1,6-glucans and β-1,3-glucans in the cell wall of *C. albicans*, cells were grown as described above. Then, 1 mL of culture was fixed at ambient temperature in the dark (1-2 h) with paraformaldehyde 3.8% (PFA, Electron Microscopy Sciences). Cells were then centrifuged 3 min at 8000 g and washed 3 times with 500 µL PBS. In each step, cells were resuspended vigorously to separate the cells and disperse aggregates. Then, cells were resuspended vigorously in 500 µL of PBS and 5% goat serum (Sigma) and then 20 µL were added to each well of a slide (Diagnostic Microscope Slides, 8 well 6 mm, ThermoScientific) coated poly-L-lysine 0.1% and incubated 1 h at ambient temperature to permit cell adhesion. Liquid was removed and PBS 1X and 5% goat serum was added again and incubated during 1 h at ambient temperature. The liquid was aspirated and 20 µL of first antibody was added in PBS 1X and 5% goat serum overnight at 4°C (dilution 1/100 for polyclonal anti-β-1,6-glucan produced in rabbit, 1/250 for monoclonal anti-β-1,3-glucan (named 5H5) produced in mouse and kindly provided by N. Nifantiev[120]). Wells were washed three times with PBS 1X and 5% goat serum. The second antibody was then added in PBS 1X and 5% goat serum 1h at ambient temperature (dilution 1/200, Alexa Fluor™ 488 goat anti-mouse IgG (AF488, molecular probes) or fluorescein goat anti-rabbit IgG (FITC, Invitrogen)). Wells were washed 3 times with PBS and 5% (v/v) goat serum and once with PBS. Then, one drop of Fluoromount-G (Invitrogen) was added to each well, wells were covered with a coverslip N°1.5 (Menzel-Gläser), and polymerization was done during 48 h. Cells were observed on an EVOS FL microscope (Life Technologies), with a magnification x100 objective with oil immersion.

PBMC and neutrophil isolation, stimulation by parietal fractions and cytokine quantification

Human peripheral blood mononuclear cells (PBMCs) from heathy donors were isolated by a density- gradient separation of Ficoll 400 (Eurobio, France). Isolated PBMCs were re-suspended in RPMI 1640 + GlutaMAX™ medium (Gibco), supplemented by 20% normal human sera (NHS, Normal Human Serum-Pooled, Zenbio) and seeded in each well (100 µL containing $4x10^6$ cells/mL) of 96- well microtiter plates (TPP). Cell wall fractions suspended in RPMI 1640 + GlutaMAX™ medium were then added, at a final concentration of 25 µg/mL. LPS (Sigma, 0.1 µg/mL) was used as a positive control. After 24 h incubation at 37°C in a 5% CO2 chamber (Cytoperm 2, ThermoFisher Scientific), the culture supernatants were collected and stored at −20°C until further analysis.

Neutrophils from healthy donors were isolated with EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies) according to manufacturer's instructions. Isolated neutrophils were re- suspended in RPMI 1640 + GlutaMAX™ medium (Gibco) and seeded in each well (100 µL containing $1x10^6$ cells/mL) of 24-well microtiter plates (TPP). 200 µL of NHS at 3% diluted in RPMI 1640 + GlutaMAX™ medium were the added. Cell wall fractions suspended in RPMI 1640 + GlutaMAX™ medium were then added, at a final concentration of 25 µg/mL. LPS (0.1 µg/mL) was used as a positive control. After 16 h incubation at 37°C in a 5% CO2 chamber (Cytoperm 2, ThermoFisher Scientific), the culture supernatants were collected and stored at −20°C until further analysis.

Global cytokines, chemokines, and acute phase proteins (total 36) present in the supernatants were detected with using Proteome Profiler Human Cytokines Array Kit (R&D Systems) according to manufacturer's instructions. Following, the cytokines, chemokines and acute phase proteins of interest identified through protein profiler were quantified using DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions.

Native AI and periodate oxidized AI (AI-OxP) fractions and purified β-1,6-glucans from *C. albicans* were used. AI-OxP fraction was generated by the treatment of 40 mg of AI fraction with 100 mM *m*-IO4Na (4 mL), 3 days at 4°C. The reaction was stopped by addition of 200 µL of glycerol, oxidized fraction was washed by centrifugation with water, reduced with BH4Na (10 mg/mL in 0.1 mM NaOH, overnight) then submitted to mild acid hydrolysis (AcOH 10% 100°C, 1 h) and finally washed with water and freeze-dried. Purified β-1,6-glucans were produced by digesting the AI fraction (40 mg) with LamA as described above. LamA was removed by passing the sample through a C-18 SPE cartridge (Sep-Pak classic, Waters) eluted with 5% acetonitrile and 0.1% TFA. Then, samples were purified by gel filtration chromatography (Superdex 200, 60 cm x 16 cm, Cytiva) in ammonium acetate 150 mM, pH 4 at a flow rate of 0.5 mL/min. Samples were detected by a refractive index detector (Iota2, Precision Instruments) and dialyzed against water and then freeze-dried. The fractions were quantified by the phenol-sulfuric acid colorimetric assay as previously described.

Complement activation

Complement activation was done as previously described[121]. Briefly, cell wall fraction from *C. albicans*: AI, AI-OxP and purified β-1,6-glucans were coated on 96-well microtiter plates (100 µL/well) at three different concentrations 50 µg, 25 µg and 12.5 µg per well in 50 mM bicarbonate buffer, pH 9.6, overnight at ambient temperature. Then, supernatants were discarded, and wells were blocked with PBS 1X/BSA 1% for 1h at ambient temperature. Then 8 µL of NHS and 92 µL of Gelatin veronal buffer (GVB, 5 mM barbital, 145 mM NaCl, 0.1% gelatin, pH 7.4) supplemented by MgCl2 (0.5 mM) and CaCl2 (0.15 mM) were added, incubated for 1 h, and then washed three times with PBS 1X/Tween-20 0.05%. Complement activation was measured by quantifying deposited C3b upon adding the wells with mouse monoclonal anti-human C3b antibody (MA1-82814, ThermoFisher Scientific, diluted 1:1,000) diluted in PBS 1X/BSA 1% and incubating 1 h at ambient temperature, washing the wells with PBS 1X/Tween 20 0.05%, adding the wells with peroxidase-conjugated secondary anti-mouse IgG antibodies (Sigma-Aldrich, diluted 1:1,000) and incubating for 1h at ambient temperature. After washing, 100 µL of substrate solution (TMB, BioFX; 100 µL/well) was added and the reaction was stopped with 50 µL of 4% H2SO4. The absorbance was read at 450 nm after subtraction of reference wavelength at 540 nm using microplate reader (Infinite m200 pro, TECAN).

Generation of mutants by transient CRISPR/Cas9

Homozygous null mutants (*kre6*Δ/Δ*; skn1*Δ/Δ; *kre62*Δ/Δ *; skn2*Δ/Δ *; kre6/skn1*Δ/Δ*; kre6/skn1/kre62/skn2*Δ/Δ*)* were generated in *C. albicans* SC5314 using established transient CRISPR-Cas9 methods[122,123]. A sgRNA cassette was constructed by PCR, first by synthesising the SNR52 promoter (using primers SNR52/F and SNR52/R/GOI) and the sgRNA scaffold (with the primers

sgRNA/F/GOI and sgRNA/R), using plasmid pV1093 as a template[122]. These two products were fused by PCR, followed by nested PCR with primers SNR52/N and sgRNA/N. The *CaCAS9* cassette was amplified from the plasmid pV1093 with primers CaCas9/F and CaCas9/R. Repair templates (RT), which contained the *SAT1*-Flipper marker and harboured 80 bp homology to the 5' and 3' ends of the target gene, were amplified from pSFS2A[124]. The RT containing the hygromycin resistance gene *HygB* was amplified from pCrispr-gRNA1/hygR, derived from plasmid pV1090[122] as follows: a *BspHI* fragment carrying the *HygB* marker was cut from pYM70[125], the ends filled in with the Klenow enzyme and the fragment ligated into pV1090 digested with *StuI* and *BglII* and treated with the Klenow enzyme. PCR reactions to amplify sgRNA and CaCas9 were carried out using Phusion High-Fidelity DNA polymerase (NEB), and Q5 polymerase (NEB) was used for RT amplification, according to the manufacturer's instructions. All PCR products were cleaned-up and concentrated with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). PCR products (3 µg of CaCAS9 cassette, 1 µg of sgRNA cassette and 3 µg of the relevant RT) were transformed into *C. albicans* SC5314 using the lithium acetate method[126]. Transformants were selected on YPD containing 150 µg/mL nourseothricin (Jena Bioscience) or YPD with 600 µg/mL hygromycin (Sigma). Disruption of both alleles of the target locus and integration of the RT were confirmed by PCR after DNA extraction (MasterPure Yeast DNA Purification Kit, Lucigen). The *SAT1* gene was removed with the flipper system. Briefly, the transformants were cultivated overnight in 3 mL YP with 2% maltose at 30°C. Then, the culture was diluted to 250 cells/mL in sterile water and 200 µL were plated on YPD with 25 µg/mL nourseothricin and incubated 1-2 days at 30°C. The smaller colonies were replicated on YPD vs. YPD + 150 µg/mL nourseothricin. Transformants that grew only YPD were checked by PCR using primers Flanking_F/GOI and Flanking_R/GOI. The primers used for the construction and PCR check of these mutants are described in **Table S3**. Deletions were validated by PCR (**Fig. S9**).

The quadruple mutant *kre6/skn1/kre62/skn2*Δ/Δ was complemented by integrating a StuI-linearized plasmid bearing the *KRE6* gene under the control of the constitutive promoter *ACT1* at the *RPS1* locus. This plasmid was obtained using the Gateway™ technology (Invitrogen™) as described previously[127]. *KRE6* was PCR amplified on SC5314 genomic DNA with primers KRE6-FWD and KRE6-REV; the fragment was cloned in pDONR207[128] with the Invitrogen™ Gateway BP clonase, and then transferred to the destination vector CIp10-P*ACT1-SAT1*[129] using the Invitrogen™ Gateway LR clonase. Successful cloning into the destination vector was confirmed by sequencing. Transformants were selected on YPD+100 µg/mL nourseothricin. Plasmid integration at the *RPS1* locus was checked by colony PCR using CIpUL and CIpSAT.

Phenotypic analyses

Kinetic curves were generated in 96 well plate (TPP) in SD medium, 30°C, at initial OD620 nm= 0.01 using a Tecan Sunrise absorbance microplate reader. Optical density was measured every 10 min during 80 h with Magellan™ Software. Doubling time was calculated from growth curves with GraphPad Prism software by generating a nonlinear regression (logistic curve) to determine the *k* coefficient, and then calculated as doubling time = ln(2)/k.

Spot test drug sensitivity assays were performed on complete SD medium agar plates containing either Congo Red (50 µg/mL), Calcofluor White (50 µg/mL), tunicamycin (1 µg/mL), nikkomycin (32 µg/mL) or caspofungin (0.5 µg/mL). Cell were precultured for 24 h in SD medium liquid, 30°C, and then diluted to OD600 nm=1 in sterile water, and then 10-fold serially diluted. Samples of 5 µL of each concentration were spotted onto agar plates. Pictures of plates were taken after 24 h, 48 h, 72 h and 6 days with a Phenobooth+ Colony counter (Singer Instruments). To observe macroscopic filamentation, 5 µL (OD600 nm=1) were spotted on YNB 2%GlcNAc, buffered at pH 7.2 with 100 mM MOPS agar, at 37°C, for 6 days. Pictures were taken using an I-phone camera.

To observe hyphal growth, cells were grown overnight in SD liquid 30°C, then diluted to OD600 nm=0.2 in 1 mL of 24 well plate (TPP) and growth for 6 h either in SD, 30°C (control, no-filamentation) or in YNB 2%GlcNAc, buffed at pH 7.2 with 100 mM MOPS, at 37°C (inducing

filamentation). Cells were then observed with an Olympus IX 83 microscope and images were captured with a Hamamatsu ORCA Flash cooled CCD camera, using the cellSens imaging software.

Statistical analyses

GraphPad Prism 10 software was used for statistical analyses. Data were generated from at least three independent biological replicates and then expressed as means ± standard deviation. Specific tests are indicated in the figure legends. Normally distributed datasets (QQ plot or Shapiro-Wilk test), were compared with ordinary one-way ANOVA (Dunnett's or Tukey's multiple comparisons test) or two-tailed unpaired *t*-test. For immunology data, measurements were paired/dependant as the same donors were exposed to different cell wall fractions, thus we applied the nonparametric test for non- normally distributed data: Friedman test (with Dunn's multiple comparisons test). P-values (P) < 0.05 were considered as significant and represented on graphs as followed: *, P < 0,05; **, P < 0,01; ***, P < 0,001; ****, P < 0,0001; ns : non-significant.

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Additional information

Author contributions

C.B., V.A., S.B.B, N.G. and T.F. designed experiments and analyzed data. C.B. performed most of the experiments. I.V. and M.C. constructed strains. C.S. and T.M. designed and performed electron microscopy experiments. I.G. performed NMR experiments and analysis. NG provided essential strains and materials. C.B., V.A., C.dE., N.G. and T.F wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Supplementary figures

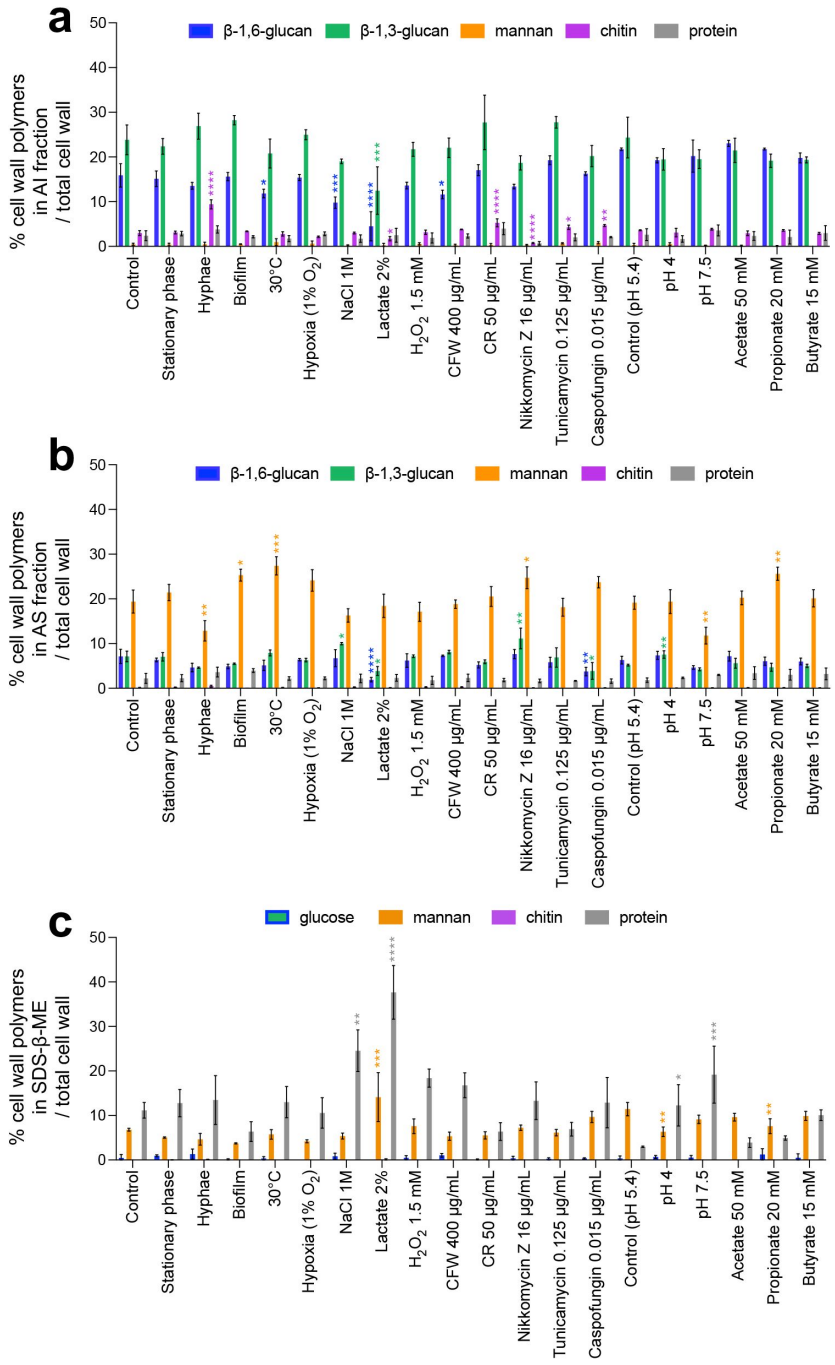


Figure S1

Global cell wall composition produced by *C. albicans* in different environmental conditions.

Results are represented as the percentage of each polymer on total cell wall in AI fraction (**a**), AS fraction (**b**) and SDS-β-ME fraction (**c**). Means and standard deviations were obtained from three independent replicate experiments. All data were compared to the control conditions and were analysed using one-way ANOVA with Dunnett's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$.

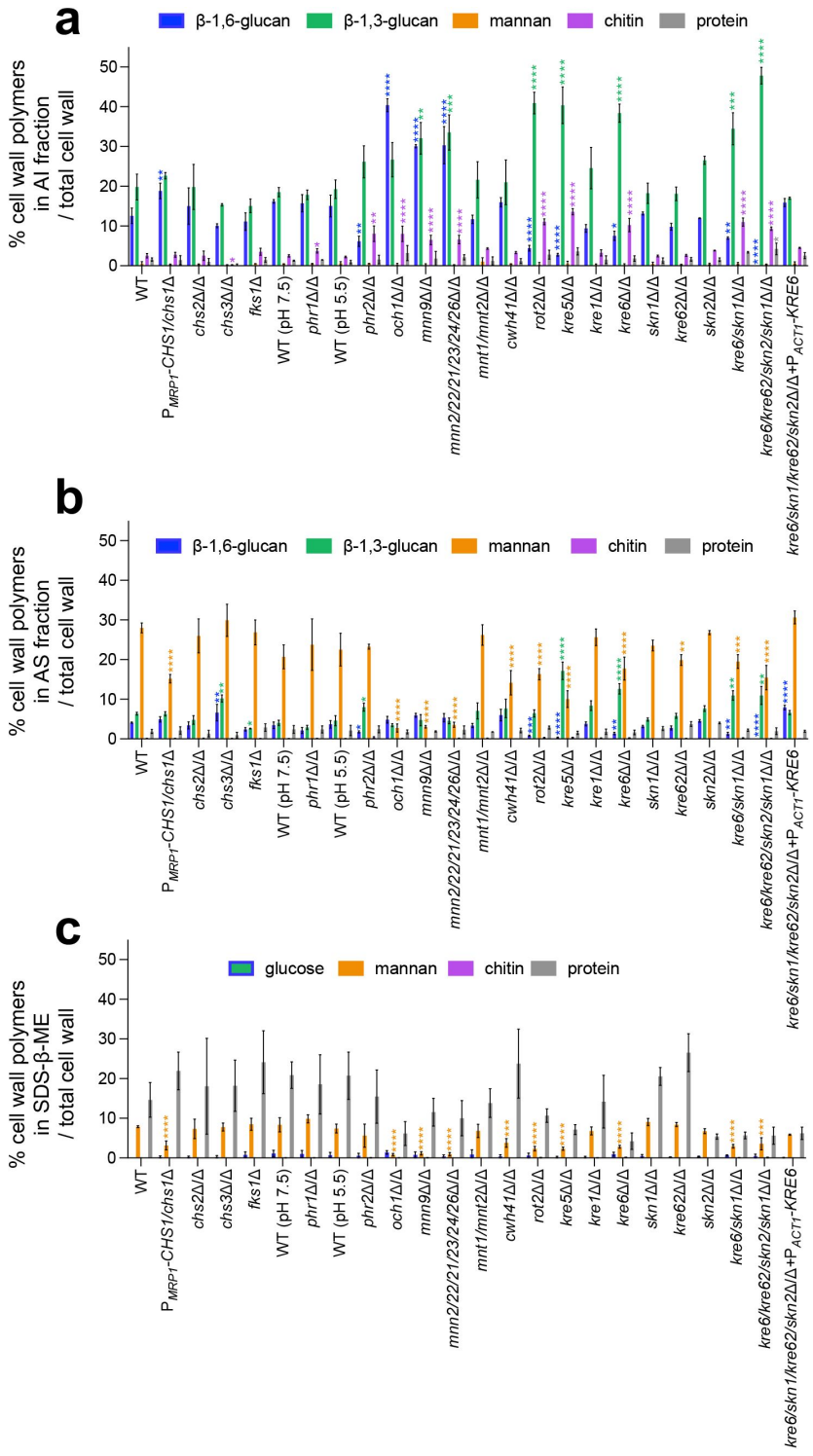


Figure S2

Cell wall composition of *C. albicans* mutants.

Results are represented as the percentage of each polymer on total cell wall in AI fraction (**a**), AS fraction (**b**), and SDS-β-ME fraction (**c**). Means and standard deviations were obtained from three independent replicate experiments. All data were compared to the parental strain SC5314 and wereanalysed using one-way ANOVA with Dunnett's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$.

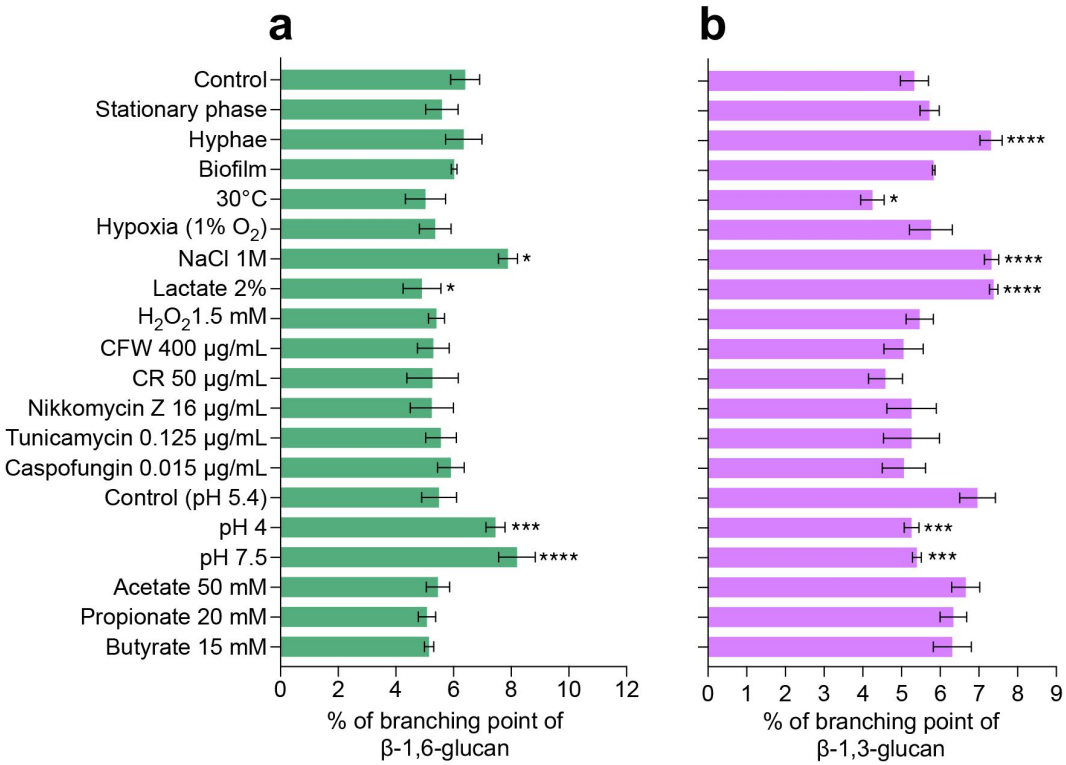


Figure S3

Branching rates of β-1,6-glucans and β-1,3-glucans produced by *C. albicans* under various environmental conditions.

a, Branching rate of β-1,6-glucans from AI fraction. The branching rate was estimated by HPAEC after digestion by an endo-β-1,6-glucanase.

b, Branching rate of β-1,3-glucans from AI fraction, The branching rate was estimated by HPAEC after digestion by an endo-β-1,3-glucanase.

Means and standard deviations from three independent replicate experiments are shown. All data were compared to the control conditions and were analysed using one-way ANOVA with Dunnett's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$.

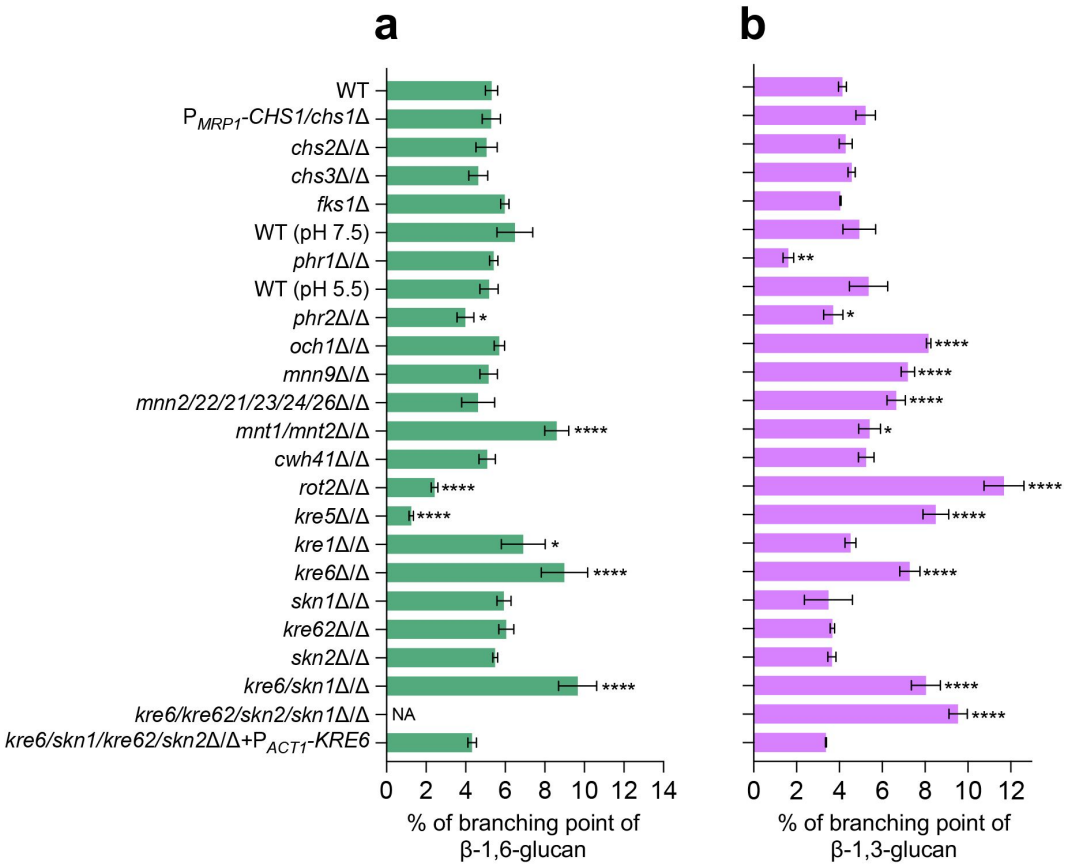


Figure S4

Branching rates of β-1,6-glucans and β-1,3-glucans produced by different cell wall mutants of *C. albicans*.

a, Branching rate of β-1,6-glucans from AI fraction. The branching rate was estimated by HPAEC after digestion by an endo-β-1,6-glucanase.

b, Branching rate of β-1,3-glucans from AI fraction. The branching rate was estimated by HPAEC after digestion by an endo-β-1,3-glucanase.

Means and standard deviations from three independent replicate experiments are shown. All data were compared to the control conditions and were analysed using one-way ANOVA with Dunnett's multiple comparisons test: *, $P < 0,05$; **, $P < 0,01$; ***, $P < 0,001$; ****, $P < 0,0001$; NA: non- applicable.

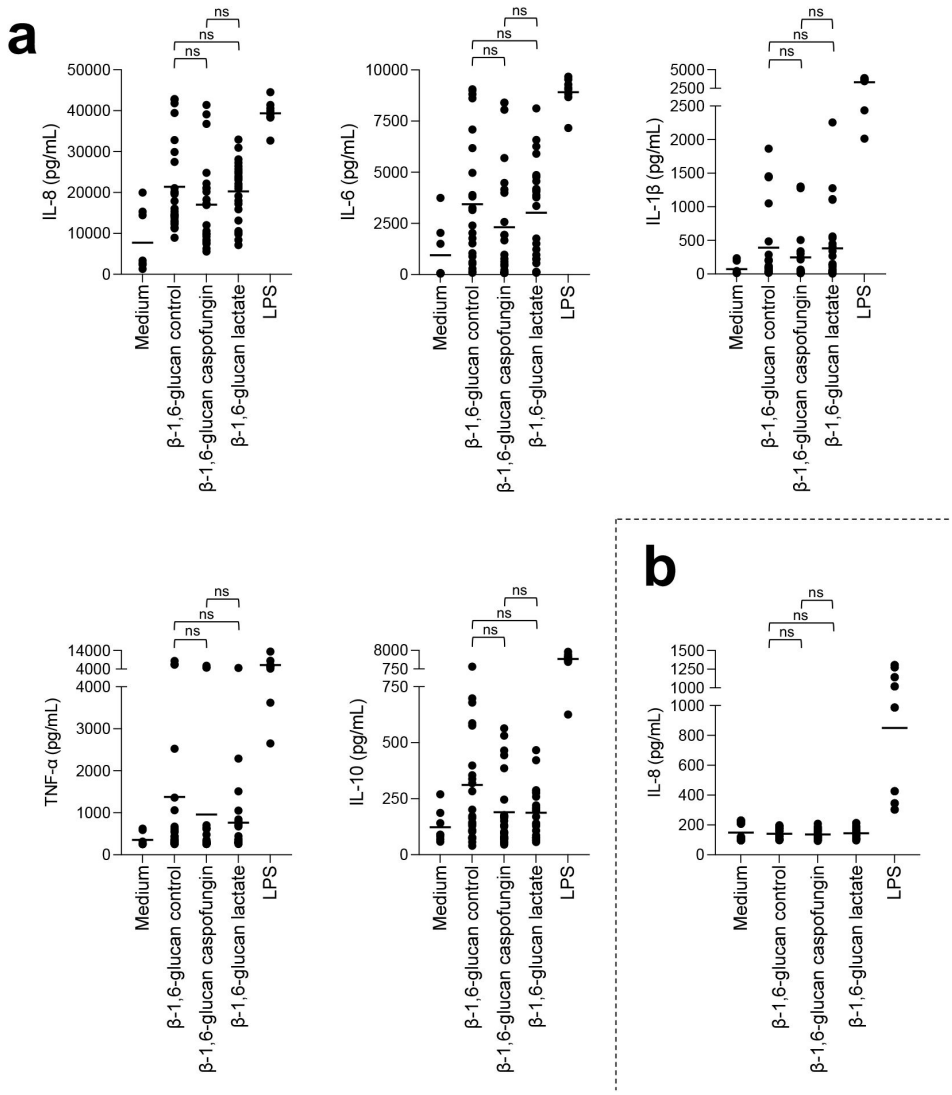


Figure S5

Stimulation of PBMCs and neutrophils *in vitro* by β-1,6-glucan with different size from *C. albicans*.

Cytokines, chemokines or acute phase proteins (IL-8, MCP-1, IL-6, MIP-1β, IL-1β, TNF-α, RANTES, C5a, IL-10) concentrations in culture supernatants of PBMCs (**panel a**) or neutrophils (**panel b**) stimulated by different β-1,6-glucans from *C. albicans* at 25 µg/mL or LPS (positive control, 0.1 µg/mL). β-1,6-glucans were isolated from cell wall AI fraction of *C. albicans* grown either at 37°C in SD medium (control, β-1,6-glucan size= 58 kDa), or in the presence of caspofungin at sublethal concentration 0.015 µg/mL (β-1,6-glucan size= 70 kDa) or in the presence of 2% lactate as sole carbon source (β-1,6-glucan size= 19 kDa). PBMCs and neutrophils were isolated from healthy human donors (n=8). Three independent batches of the different fractions were used. Means are represented and data were analyzed using nonparametric Friedman test with Dunn's multiple comparisons: ns: non-significant.

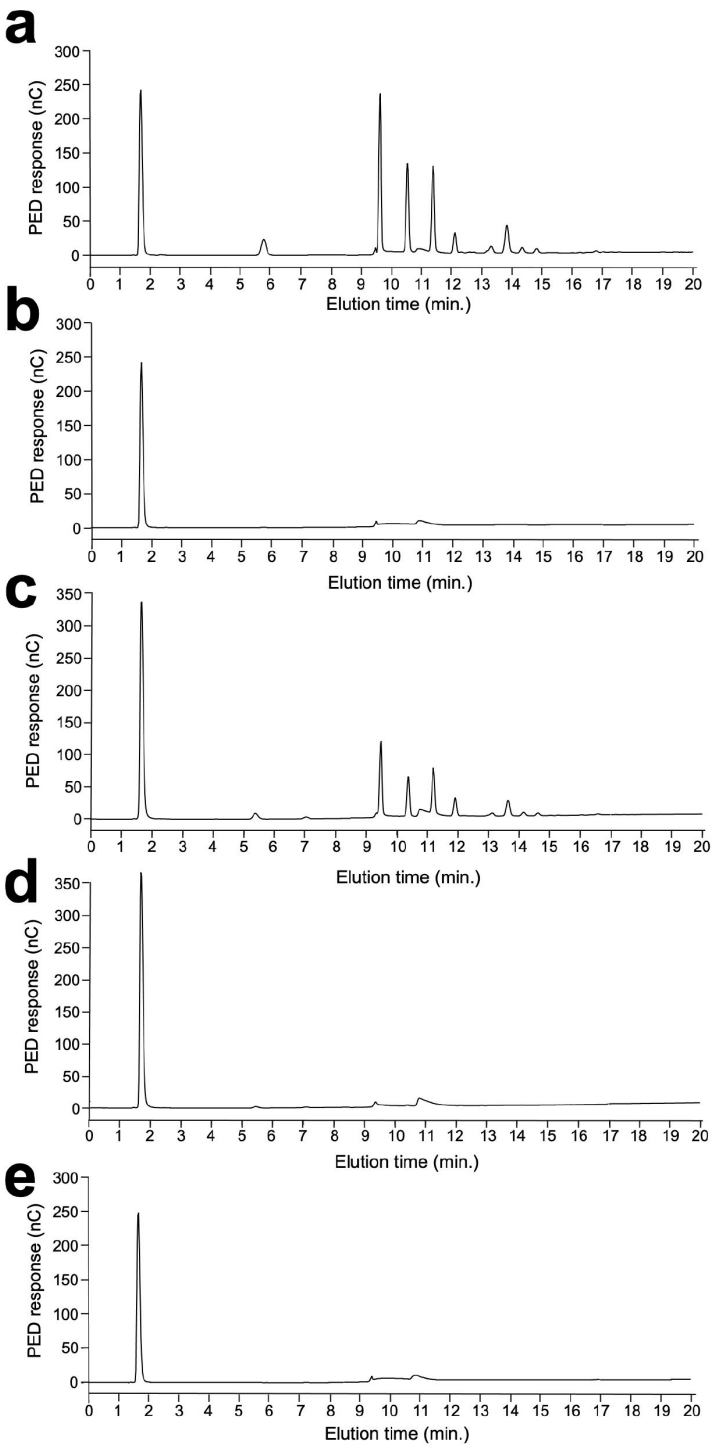


Figure S6

Absence of β-1,6-glucans in the cell wall of the quadruple *kre6/kre62/skn2/skn1*Δ/Δ mutant.

HPAEC analysis of oligosaccharides released by the endo-β-(1,6)-glucanase digestions of AI fraction of WT (**a**), AI fraction of *kre6/kre62/skn2/skn1*Δ/Δ (**b**), AS fraction of WT (**c**), AS fraction of *kre6/kre62/skn2/skn1*Δ/Δ (**d**) and water (**e**). Experiments were performed in triplicates. *PED*, pulsed electrochemical detector; *nC*, nanocoulomb.

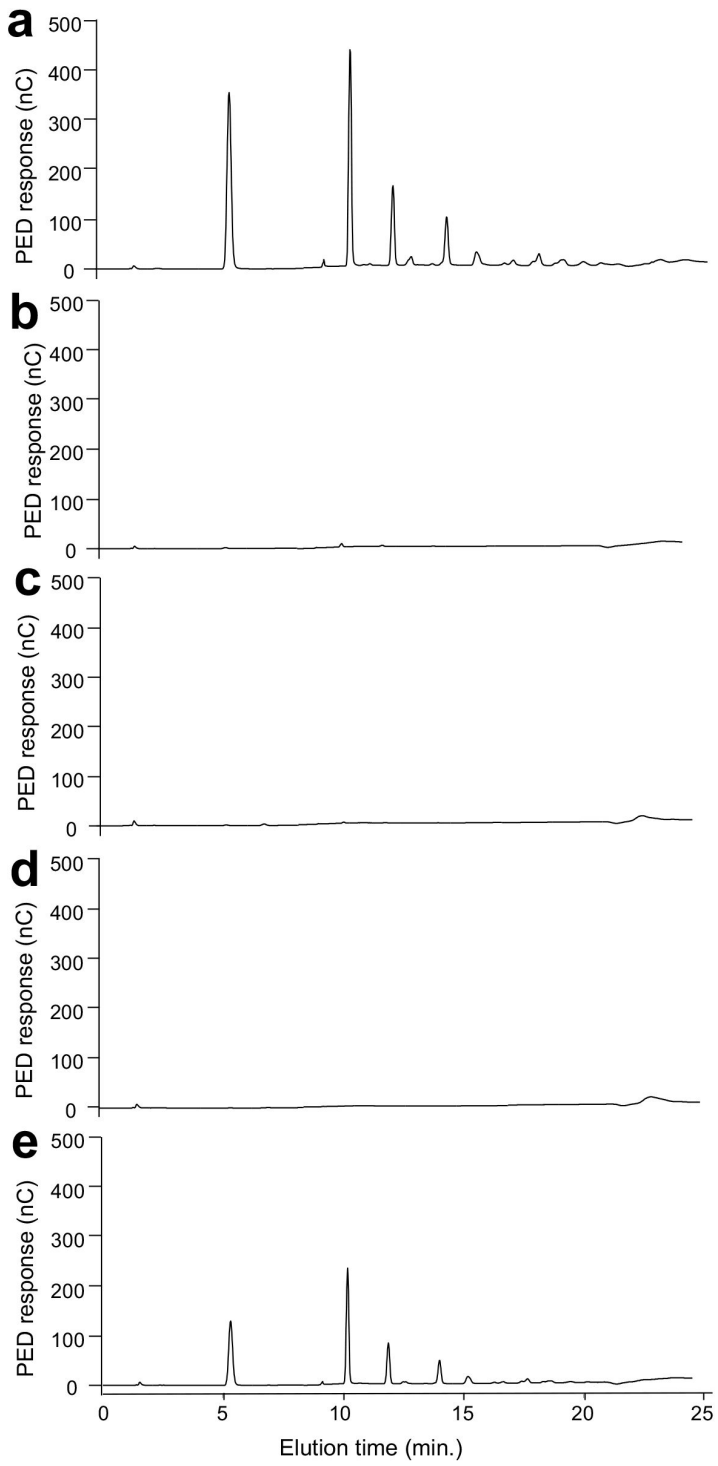


Figure S7

Cell disruption is essential to eliminate glycogen in AI and AS fractions.

HPAEC analysis of oligosaccharides released by α-amylase enzymatic digestion of AI and AS fractions.
(**a**) Control: glycogen, (**b**) AI fraction obtained after biomass cell disruption, (**c**) AI fraction from biomass with no cell disruption, (**d**) AS fraction obtained after biomass cell disruption, (**e**) AS fraction from biomass with no cell disruption. *PED*, pulsed electrochemical detector; *nC*, nanocoulomb.

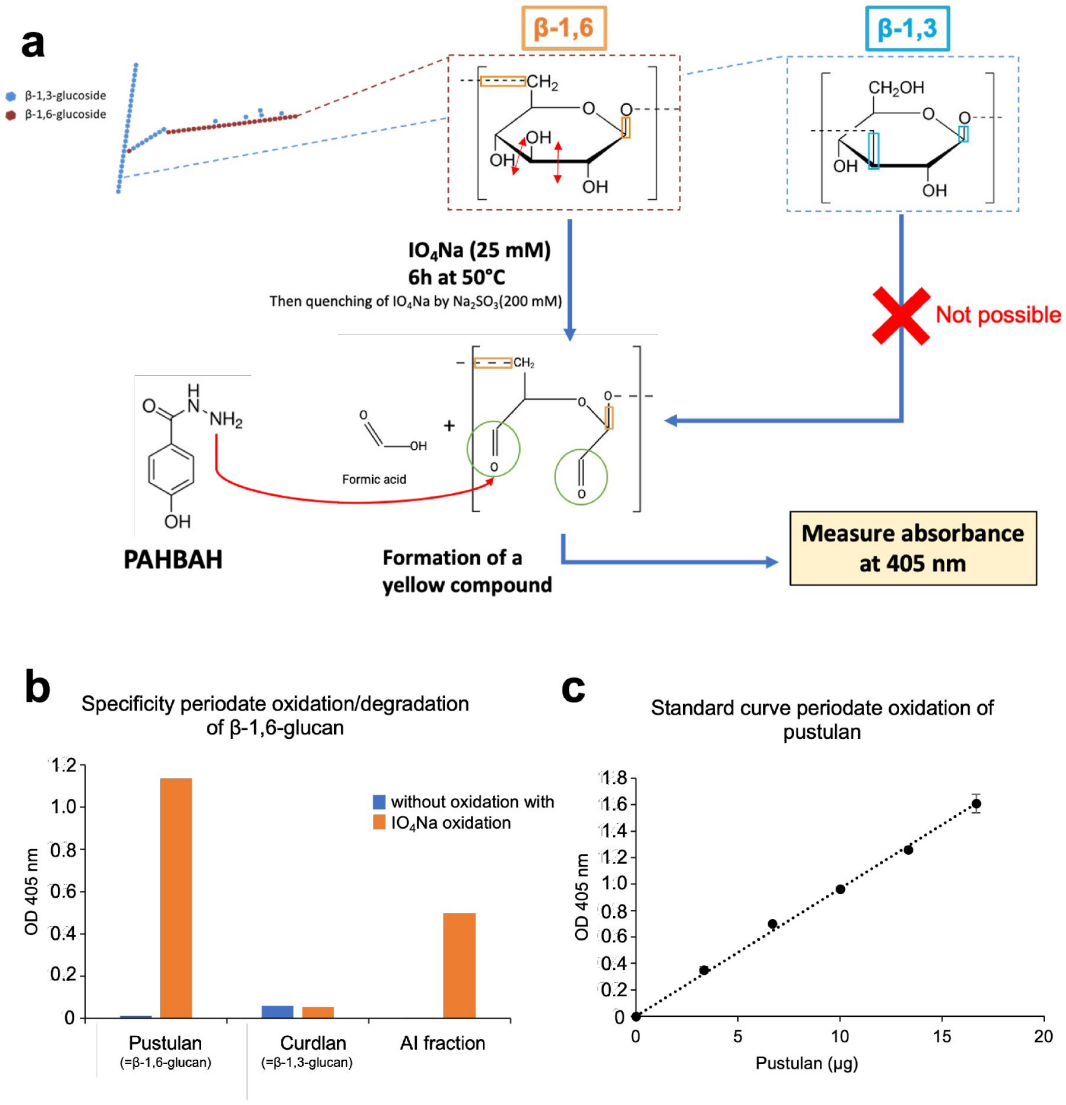
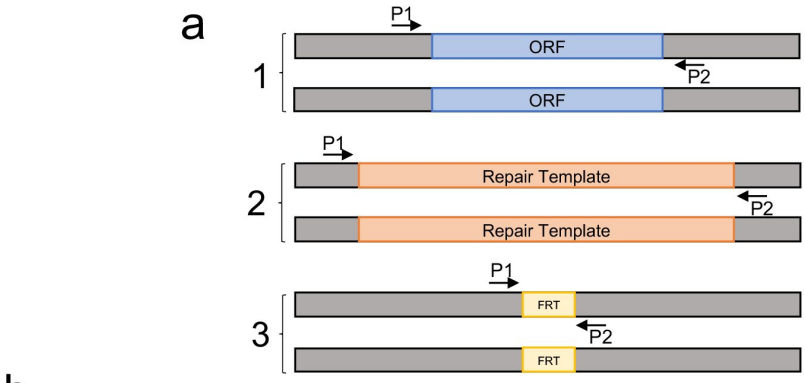


Figure S8

Quantification methods of β-1,6-glucans in AI fractions.

a, The specific oxidation of β-1,6-glucans of the AI fraction by periodate was used for quantification. Briefly, IO4Na splits bonds between vivinal carbons bearing hydroxyl groups (only present in β-1,6- glucoside), which leads to the formation of aldehydes, which can react with 4-Hydroxybenzhydrazide (PAHBAH) to form a yellow compound measurable by absorbance at OD=405 nm.
b, Specificity of the periodate oxidation method for β-1,6-glucans (pustulan). The method is specific for β-1,6-glucans (pustulan and AI fraction) and inactive on β-1,3-glucans (curdlan).
c, Linearity of β-1,6-glucan assay after periodate oxidation. We showed that the response of the method described in (a) is proportional from 0 to 20 µg of pustulan.



b

PCR band expected (in bp)	ORF=*KRE6*			ORF=*KRE62*			ORF=*SKN2*			ORF=*SKN1*		
	1	2	3	1	2	3	1	2	3	1	2	3
WT	2784			2307			2671			2483		
kre6Δ/Δ	2784	2642										
kre62Δ/Δ				2307	4592	506						
skn2Δ/Δ							2671	4668	582			
skn1Δ/Δ										2483	4423	337
kre6/skn1Δ/Δ	2784	2642								2483	4423	337
kre6/kre62/skn2/skn1Δ/Δ	2784	2642		2307	4592	506	2671	4668	582	2483	4423	337

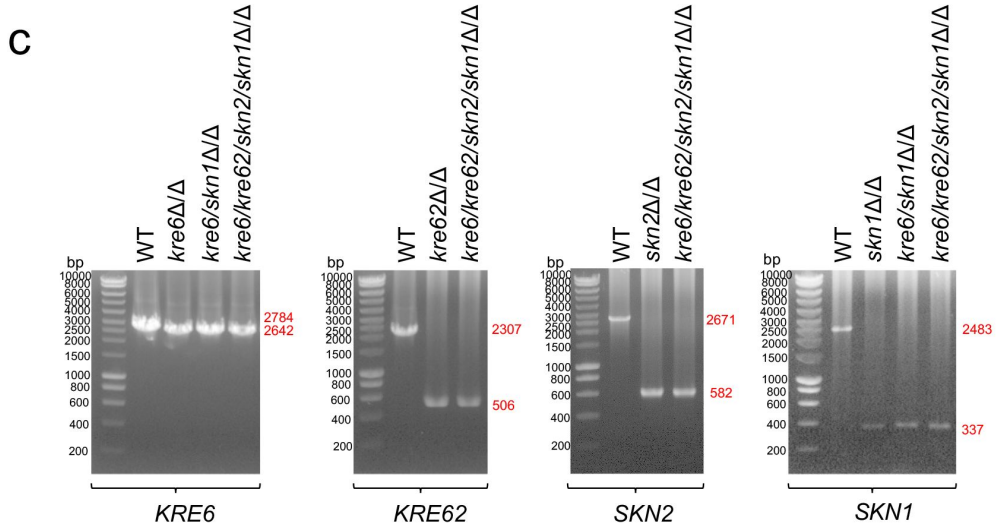


Figure S9

Control PCR control of mutants obtained in this study.

a, Principle of PCR check done in b and c. P1 and P2 are PCR diagnostic primers. ORF = *KRE6*, *KRE62*, *SKN2* or *SKN1*. Repair Templates contain either *HygB* or a *SAT1-Flipper* cassette for selection. FRT correspond to the scar after *SAT1-Flipper* cassette excision. **b**, Table of expected PCR bands according to mutant. **c**, PCR bands obtained.

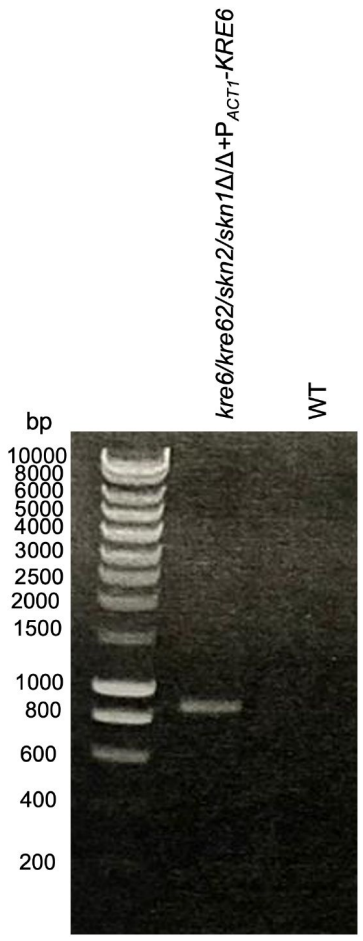


Figure S10

Control PCR of the quadruple mutant complemented for *KRE6 (kre6/kre62/skn2/skn1*Δ/Δ+P*ACT1-KRE6)*

KRE6 was reintegrated at the *RPS1* locus under the control of *ACT1* promoter, using *SAT1* as a selection marker.

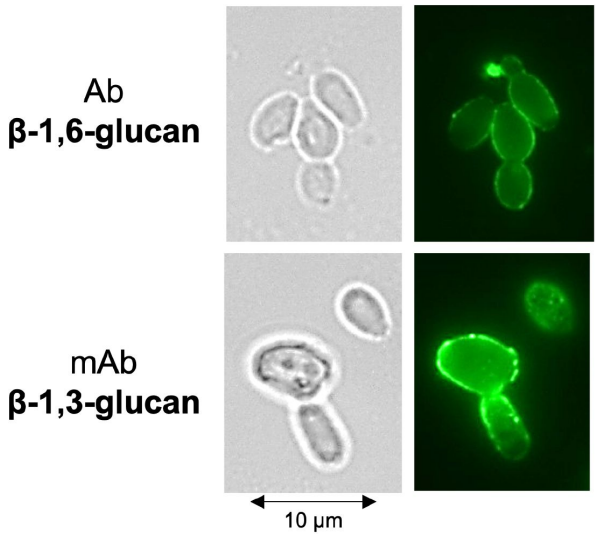
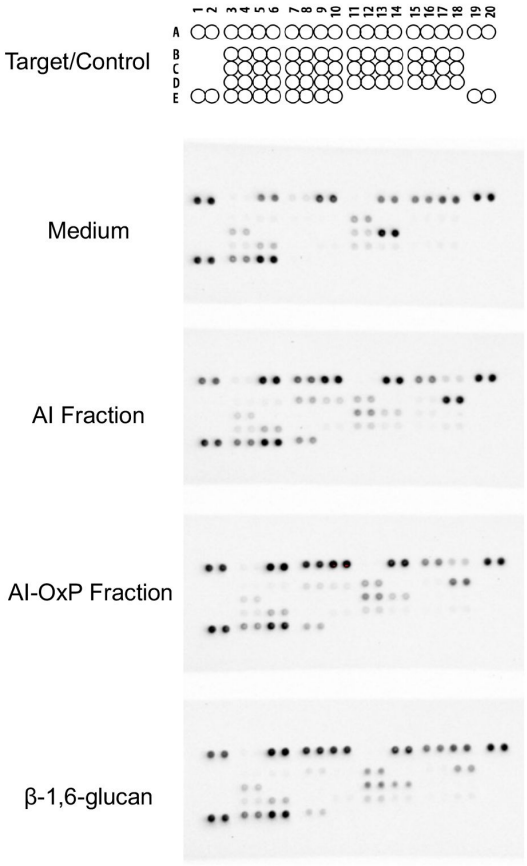


Figure S11

Exposure of β-1,6-glucans and β-1,3-glucans at the cell surface of *C. albicans* SC5314.

Cells were cultured in SD at 37°C. β-glucan exposure was detected by a polyclonal rabbit anti-β-1,6- glucan serum (top panel) and or monoclonal anti-β-1,3-glucan antibody (bottom panel).

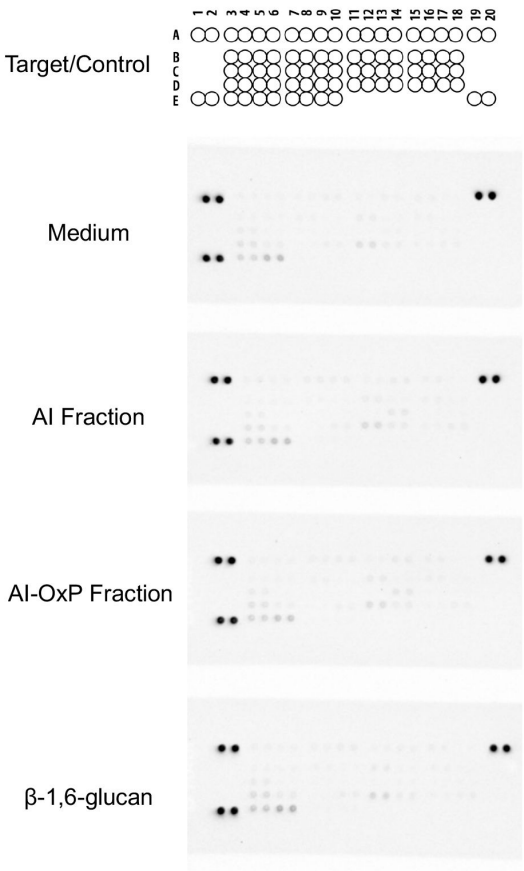


Target/Control	Coordinate		Target/Control	Coordinate
Reference Spots	A1, A2, A19, A20, E1, E2		IL-2	C5, C6
Negative Control	E19, E20		IL-4	C7, C8
CCL1/I-309	A3, A4		IL-5	C9, C10
CCL2/MCP-1	A5, A6		IL-6	C11, C12
MIP-1α / MIP-1β	A7, A8		IL-8	C13, C14
CCL5/RANTES	A9, A10		IL-10	C15, C16
CD40 Ligand /TNFSF5	A11, A12		IL-12 p70	C17, C18
Complement Component C5/C5a	A13, A14		IL-13	D3, D4
CXCL1/GR0α	A15, A16		IL-16	D5, D6
CXCL10/IP-10	A17, A18		IL-17A	D7, D8
CXCL11/I-TAC	B3, B4		IL-17E	D9, D10
CXCL12/SDF-1	B5, B6		IL-18/ IL-1F4	D11, D12
G-CSF	B7, B8		IL-21	D13, D14
GM-CSF	B9, B10		IL-27	D15, D16
ICAM-1/CD54	B11, B12		IL-32α	D17, D18
IFN-γ	B13, B14		MIF	E3, E4
IL-1α/IL-1F1	B15, B16		Serpin E1 /PAI-1	E5, E6
IL-1β/IL-1F2	B17, B18		TNF-α	E7, E8
IL-1ra/IL-1F3	C3, C4		TREM-1	E9, E10

Figure S12

Human proteome profiler done with culture supernatant from PBMCs stimulated with *C. albicans* cell wall fractions.

On the left, membrane blots obtained after incubation with supernatants from PBMCs cultures stimulated by different *C. albicans* cell wall fractions: AI, AI-OxP, β-1,6-glucans. Incubation with the culture medium was used as a control (top). On the right, coordinate of each protein (cytokines, chemokines, acute phase proteins) detected on the membranes. The experiment was performed once using a pool of 24 supernatants from the stimulation of PBMCs isolated from 8 healthy donors, each stimulated with 3 independent batches of fractions.



Target/Control	Coordinate		Target/Control	Coordinate
Reference Spots	A1, A2, A19, A20, E1, E2		IL-2	C5, C6
Negative Control	E19, E20		IL-4	C7, C8
CCL1/I-309	A3, A4		IL-5	C9, C10
CCL2/MCP-1	A5, A6		IL-6	C11, C12
MIP-1α / MIP-1β	A7, A8		IL-8	C13, C14
CCL5/RANTES	A9, A10		IL-10	C15, C16
CD40 Ligand /TNFSF5	A11, A12		IL-12 p70	C17, C18
Complement Component C5/C5a	A13, A14		IL-13	D3, D4
CXCL1/GR0α	A15, A16		IL-16	D5, D6
CXCL10/IP-10	A17, A18		IL-17A	D7, D8
CXCL11/I-TAC	B3, B4		IL-17E	D9, D10
CXCL12/SDF-1	B5, B6		IL-18/ IL-1F4	D11, D12
G-CSF	B7, B8		IL-21	D13, D14
GM-CSF	B9, B10		IL-27	D15, D16
ICAM-1/CD54	B11, B12		IL-32α	D17, D18
IFN-γ	B13, B14		MIF	E3, E4
IL-1α/IL-1F1	B15, B16		Serpin E1 /PAI-1	E5, E6
IL-1β/IL-1F2	B17, B18		TNF-α	E7, E8
IL-1ra/IL-1F3	C3, C4		TREM-1	E9, E10

Figure S13

Human proteome profiler done with culture supernatant from neutrophils stimulated with *C. albicans* cell wall fractions.

On the left, membrane blots obtained after incubation with supernatants from neutrophils cultures stimulated by different *C. albicans* cell wall fractions: AI, AI-OxP or β-1,6-glucans; incubation with the culture medium was used as a control (top). On the right, coordinate of each protein (cytokines, chemokines, acute phase proteins) detected on the membranes. The experiment was performed once using a pool of 24 supernatants from the stimulation of neutrophils isolated from 8 healthy donors, each stimulated with 3 independent batches of fractions.

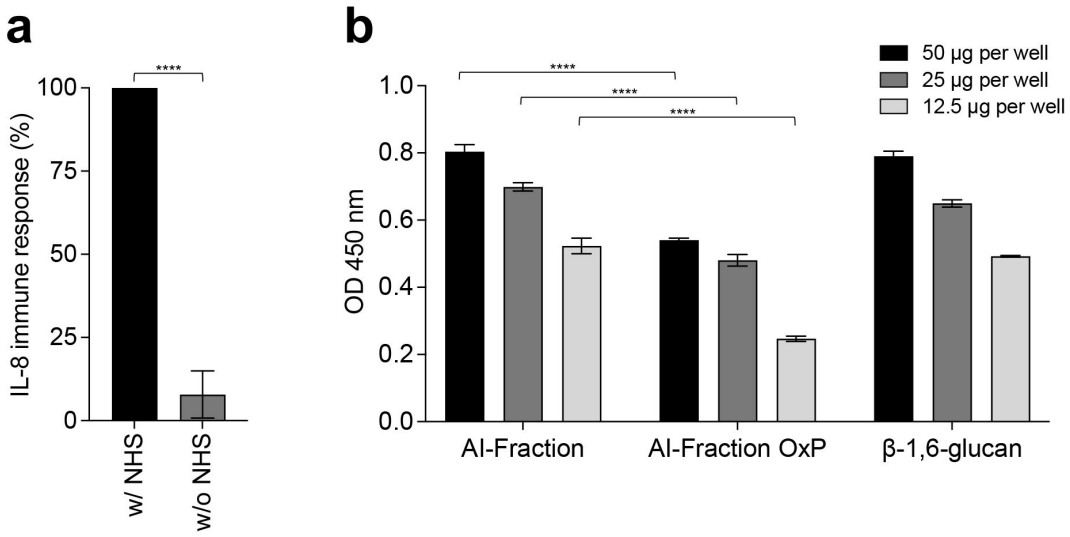


Figure S14

β-1,6-glucan from *C. albicans* activates complement system.

a, Normal human serum (NHS) enhances the immunostimulatory capacity of β-1,6-glucan from *C. albicans.* PBMCs isolated from healthy human donors (n=2) were stimulated with three independent batches of β-1,6-glucan at 25 µg/mL with (w/) or without (w/o) NHS (10%). Immune response was analyzed by measuring IL-8 released into the culture medium. Means are represented and data were analyzed with an unpaired parametric t-test: ****, $P < 0{,}0001$.

b, Complement factor C3b binds to β-1,6-glucan purified from *C. albicans* cell wall. Three cell wall fractions (AI, AI-OxP and β-1,6-glucan) from *C. albicans* were coated on microtiter plates at 50 µg, 25 µg or 12.5 µg per well. Human normal serum, diluted in Gelatin-Veronal Buffer (GVB), was added to activate complement pathways. The amount of deposited C3b on each fraction (=level on complement activation) was determined by using anti-human C3b and peroxidase-conjugated anti- mouse IgG antibodies. 3,3',5,5'-Tetramethylbenzidine (TMB) was used as the peroxidase substrate and the reaction was stopped with 4% H2SO4 and optical density (OD) was measured at 450 nm.

The experiment was done with three independent batches of each cell wall fractions. Blank value was subtracted from the values presented. Statistical analyses were performed with one-way ANOVA with Tukey's multiple comparisons test: ****, $P < 0{,}0001$.

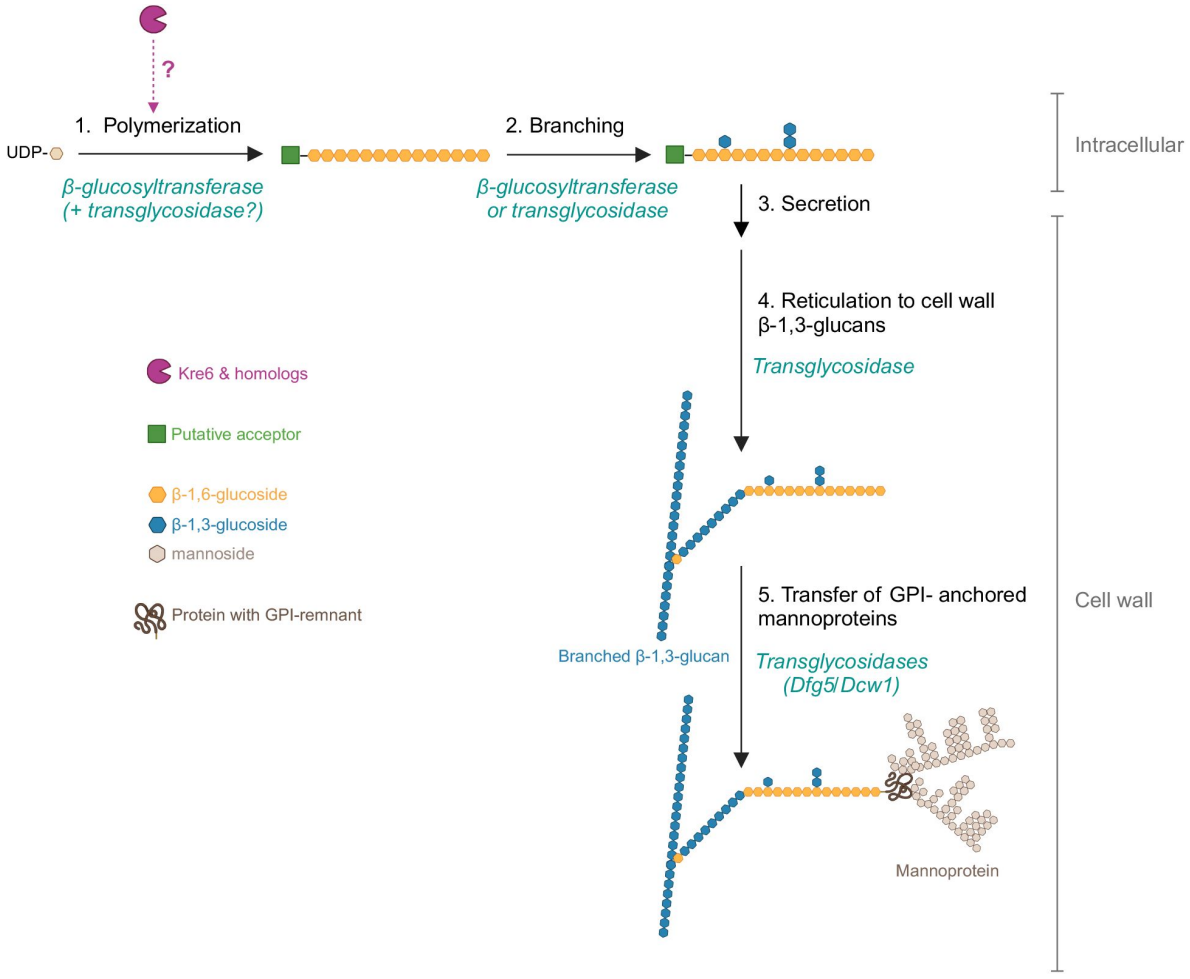


Figure S15

A model for β-1,6-glucan biosynthetic pathway and putative role of Kre6 family members in this process in yeast.

The cellular location of β-1,6-glucan synthesis in yeast is still unknown. We assume that synthesis begins intracellularly with the polymerisation of linear β-1,6-glucan chain (step 1), which requires a β-glucosyltransferase and UDP-glucose as a donor[33,92] and a putative acceptor (sugar, protein, lipid). Our data (**Fig. 3g**, S6) suggest that Kre6 and its homologues (Kre62, Skn1, Skn2) act at this stage, but their function remains unknown. Two proposed hypotheses are: 1) Kre6 family members are β- glucosyltransferases and 2) they have glycosylhydrolase and transglycosidase activity essential for polymerisation. Step 2 is the branching of nascent β-1,6-glucan where glucosides and laminaribiosides are added to form side chains. The enzymes (β-glucosyltransferase or transglycosidase) involved in this branching remain unknown. According to our data, members of the Kre6 family are not involved in branching (**Fig. 3i**). Next, the polysaccharide is secreted (step 3) and then cross-linked to β-1,3-glucans in the cell wall space by an unknown transglycosidase (step 4). The transfer of GPI-anchored proteins onto β-1,6-glucan (step 5), leading to the formation of the outer layer of the cell wall, appears to be driven by Dfg5/Dcw1[130]. The chronology between these two cross-links (Steps 4 and 5) has not been established.

Strain name	Parental strain	Relevant genotype	Reference
SC5314	-	Clinical blood isolate	PMID: 6394964
CAF2-1	SC5314	*URA3/ura3Δ::imm434*	PMID: 8349105
CAI4	CAF2-1	*ura3Δ::imm434/ura3Δ::imm434*	PMID: 8349105
BWP17	CAI4	*ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	PMID: 10074081
SN152	CAI4	*arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434*	PMID: 15701792
cwh41Δ/Δ	CAI4	Same as CAI4 but *cwh41Δ::dp1200/cwh41Δ::dp1200 RPS1/rps1Δ::*Clp10	PMID: 17933909
rot2Δ/Δ	CAI4	Same as CAI4 but *rot2Δ::dp1200/rot2Δ::dp1200 RPS1/rps1Δ::*Clp10	PMID: 17933909
kre5Δ/Δ	SN152	Same as SN152 but *kre5Δ::leu2/kre5Δ::his1*	PMID: 20543849
kre6Δ/Δ	SC5314	*kre6Δ::HygB/kre6Δ::HygB*	This study
kre62Δ/Δ	SC5314	*kre62Δ::FRT/kre62Δ::FRT*	This study
skn2Δ/Δ	SC5314	*skn2Δ::FRT/skn2Δ::FRT*	This study
skn1Δ/Δ	SC5314	*skn1Δ::FRT/skn1Δ::FRT*	This study
kre6/skn1Δ/Δ	*kre6Δ/Δ*	*kre6Δ::HygB/kre6Δ::HygB skn1Δ::FRT/skn1Δ::FRT*	This study
kre6/kre62/skn2/skn1Δ/Δ	*kre6Δ/Δ*	*kre6Δ::HygB/kre6Δ::HygB kre62Δ::FRT/kre62Δ::FRT skn2Δ::FRT/skn2Δ::FRT skn1Δ::FRT/skn1Δ::FRT*	This study
kre6/kre62/skn2/skn1Δ/Δ+ P_{ACT1}*-KRE6*	*kre6/kre62/skn2/skn1Δ/Δ*	*kre6Δ::HygB/kre6Δ::HygB kre62Δ::FRT/kre62Δ::FRT skn2Δ::FRT/skn2Δ::FRT skn1Δ::FRT/skn1Δ::FRT RPS1/RPS1::*Clp*SAT1-*P_{ACT1}*-KRE6*	This study
kre1Δ/Δ	BWP17	Same as BWP17 but *kre1Δ::arg4/kre1Δ::his1*	Provided by Mathias Richard

Table S1

***C. albicans* strains used in this study.**

P_{MRP1}-*CHS1/chs1*Δ	CAI4	Same as CAI4 but *chs1*Δ::*hisG/chs1*Δ:pSK-*URA3*-P_{MRP1}-*CHS1*	PMID: 11251855
*chs2*Δ/Δ	CAF2-1	Same as CAF2-1 but *chs2*Δ::*hisG/chs2*Δ::*hisG-URA3-hisG*	PMID: 8636047
*chs3*Δ/Δ	CAF2-1	Same as CAF2-1 but *chs3-2::hisG/chs3-3::hisG-URA3-hisG*	PMID: 7479842
*mnt1/mnt2*Δ/Δ	CAI4	Same as CAF2-1 but *mnt1-mnt2*Δ::*hisG/mnt1-mnt2*Δ::*hisG-URA3-hisG*	PMID: 15519997
*mnn2/22/21/23/24/26*Δ/Δ	CAI4	Same as CAF2-1 but *mnn2*Δ::*dpl200/mnn2*Δ::*dpl200 mnn22*Δ::*dpl200/mnn2*Δ::*dpl200 mnn23*Δ::*dpl200/mnn23*Δ::*dpl200 mnn24*Δ::*dpl200/mnn24*Δ::*dpl200 mnn26*Δ::*dpl200/mnn26*Δ::*dpl200 mnn21*Δ::*dpl200/mnn21*Δ::*dpl200*	PMID: 23633946
*mnn9*Δ/Δ	CAI4	Same *as CAF2-1 mnn9*Δ::*hisG/mnn9*Δ::*hisG* Δ*ura3*Δ::*imm434/ura3*Δ::*imm434*	PMID: 10601199
*och1*Δ/Δ	SN152	Same as SN152 but *och1*Δ::*leu2/och1*Δ::*his1*	PMID: 20543849
*fks1*Δ	SC5314	SC5314, but *fks1/fks1*Δ	PMID: 30370375
*phr1*Δ/Δ	BWP17	Same as BWP17 but *phr1*Δ::*hisG/phr1*Δ	PMID: 7823929
*phr2*Δ/Δ	BWP17	Same as BWP17 but *phr2*Δ::*hisG/phr2*Δ::*hisG-URA3-hisG*	PMID: 9315654

Table S1 (continued)

Residue	H1, C1 $^{3}J_{H1/H2}$, $^{1}J_{H1/C1}$	H2, C2	H3, C3	H4, C4	H5, C5	H6-H6', C6
-6)-β-Glc-(1,6)-	4.486 105.88 7.9 Hz 162 Hz	3.300 76.00	3.469 78.57	3.428 72.52	3.599 77.85	4.184-3.822 71.81
-3,6)-β-Glc-(1,6)-	4.519 105.63 7.8 Hz 162 Hz	3.498 75.71	3.707 87.46	3.544 71.07	3.627 77.52	4.191-3.838 71.81
β-Glc-(1,3)-	4.701 105.77 7.9 Hz 163 Hz	3.329 76.43	3.492 78.5	3.379 72.55	3.446 78.92	3.883-3.690 63.71

Table S2

^{1}H and ^{13}C NMR resonance assignments, $^{3}J_{H1/H2}$ and $^{1}J_{H1/C1}$ coupling constants of the monosaccharide residues of cell wall β-1,6-glucan purified from the AI fraction.

Chemical shifts are expressed in ppm and coupling constants in Hz.

Primer name	Sequence (5' -> 3')	Remarks
SNR52/F	AAGAAAGAAAGAAAACCAGGAGTGAA	Forward primer for the amplification of SNR52 promoter (Min 2016)
sgRNA/R	ACAAATATTTAAACTCGGGACCTGG	Reverse primer for the amplification of sgRNA scaffold (Min 2016)
SNR52/N	GCGGCCGCAAGTGATTAGACT	Forward and reverse primers for nested PCR (for construction of sgRNA expression cassette; Min 2016)
sgRNA/N	GCAGCTCAGTGATTAAGAGTAAAGATGG	
CaCas9/F	ATCTCATTAGATTTGGAACTTGTGGGTT	Forward primer for amplifying the CaCas9 cassette (Min 2016)
CaCas9/R	TTCGAGCGTCCCAAAACCTTCT	Reverse primer for amplifying the CaCas9 cassette (Min 2016)
SNR52/R/SKN1	CGAGTCATTTCGTCTTGGTTCAAATTAAAAATAGTTTACGCAAGTC	Reverse primers for the amplification of SNR52 promoter with overlapping guide sequence of the target gene. Guide sequence in red.
SNR52/R/SKN2	TTCGATTCTAGCAACAGACTCAAATTAAAAATAGTTTACGCAAGTC	
SNR52/R/KRE62	GATATATTACTTGAATCACTCAAATTAAAAATAGTTTACGCAAGTC	
SNR52/R/KRE6	CTATAAGCTTGGAATGGTTTCAAATTAAAAATAGTTTACGCAAGTC	
sgRNA/F/SKN1	AACCAAGACGAAATGACTCGGTTTTAGAGCTAGAAATAGCAAGTTAAA	Forward primers for the amplification of sgRNA scaffold with overlapping guide sequence of the target gene. Guide sequence in red.
sgRNA/F/SKN2	AGTCTGTTGCTAGAATCGAAGTTTTAGAGCTAGAAATAGCAAGTTAAA	
sgRNA/F/KRE62	AGTGATTCAAGTAATATATCGTTTTAGAGCTAGAAATAGCAAGTTAAA	
sgRNA/F/KRE6	AAACCATTCCAAGCTTATAGGTTTTAGAGCTAGAAATAGCAAGTTAAA	

Table S3

Primers used in this study.

SAT1FLP/F/SKN1	ACTACCACTACTATTATAGAAATTTTTATCATA TATATACAATTGGCTACTAAAAACTTAAA CTTTTAATACAACTTACAGTACCGGGCCCCC CCTCGA	Primers to amplify the repair template (*SAT1*-Flipper or HygR)
SAT1FLP/F/SKN2	ATAATAGATTTTGTTTTTAAACGGATAACCCT TCCATTCCATTCTAGCAAACCAAAAGTAAA TCAAAAGAACTAACTACCGTACCGGGCCCCC CCTCGA	
SAT1FLP/F/KRE62	AATATTCAACAACTATTCTTTCTACTTCTTTTA AAGAACCCGAACTTTTTTTTTGACTTTATAA TAATTAATGTATCATTGTACCGGGCCCCCCC TCGA	
HygR/F/KRE6	GATAAGACCTCAAAATGGCGTCTCAAAGAGA GATGGAATGAATGGGATGAATCATCAAACAA GAG	
SAT1FLP/R/SKN1	AATTATATACAATGAATGAATGAATGAAAATG TCTATTTAAAAGTATATAAAAATATG TAAATATAGGGGGGTTGGTGTTGGCCGCTCT AGAACTAGTGGAT	
SAT1FLP/R/SKN2	CAGAGAACACATGAAGGGTGGTAAACTATTT TGTCAGTTTTTTTTCTCCTTTTTTTTAGT ACGTGGAGTTCGCCTTTAATGGCCGCTCTAG AACTAGTGGAT	
SAT1FLP/R/KRE62	ATCGTCTTGTTTGAGATTGTTCTTTTAACTTTT GTTCTACACTACCACCACCACCACCATC ATTGTTATTCTTGTTGTTGGGCCGCTCTAGAA CTAGTGGAT	
hygR/R/KRE6	AGTAGTAGTAGTAATAGTAGTACCATCGCCA CCTGACGTCGTATAGTGCTTGCTGTTCG	
Flanking_F/SKN1	CTACAGTTTATAGAAGTTAATACCAGTACC	
Flanking_F/SKN2	ATATCCCGCTCCATCCATTC	
Flanking_F/KRE62	AGGCTAGGCTAAGTATTAAC	
Flanking_R/SKN1	CATTATCATCAGGTCAATTTGACTCAT	
Flanking_R/SKN2	ACCACTGTCGTACAAAATTGC	
Flanking_R/KRE62	ATTACCTGATTTGGTGATGC	
screenKre6/F	ACTTGGCAACATTTGCAACA	
screenKre6/R	TCTGGGGTCACTTTCTTAAAATCCT	
ClpUL	ATACTACTGAAAATTTCCTGACTTTC	
ClpSAT	CTAGTGGATCCCCCAGATCATTATCC	
KRE6-FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTtgA TGGCGTCTCAAAGAGATTTAACTTCAAAT	Primers used for cloning KRE6 in pDONR207

Table S3 (continued)

KRE6-REV	GGGGACCACTTTGTACAAGAAAGCTGGGTcT TAACATCCAATTAAACTATTTTTAGGGAA	

Table S3 (continued)

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Reviewer #1 (Public review):

Summary:

The fungal cell wall is a very important structure for the physiology of a fungus but also for the interaction of pathogenic fungi with the host. Although a lot of knowledge on the fungal cell wall has been gained, there is lack of understanding of the meaning of ß-1,6-glucan in the cell wall. In the current manuscript, the authors studied in particular this carbohydrate in the important human-pathogenic fungus Candida albicans. The authors provide a comprehensive characterization of cell wall constituents under different environmental and physiological conditions, in particular of ß-1,6-glucan. Also, β-1,6-glucan biosynthesis was found to be likely a compensatory reaction when mannan elongation was defective. The absence of β-1,6-glucan resulted in a significantly sick growth phenotype and complete cell wall reorganization. The manuscript contains a detailed analysis of the genetic and biochemical basis of ß-1,6-glucan biosynthesis which is apparently in many aspects similar to yeast. Finally, the authors provide some initial studies on immune modulatory effects of ß-1,6-glucan.

https://doi.org/10.7554/eLife.100569.2.sa3

Reviewer #2 (Public review):

Summary:

The authors provide the first (to my knowledge) detailed characterization of cell wall b-1,6 glucan in the pathogen Candida albicans. The approaches range from biochemistry to genetics to immunology. The study provides fundamental information and will be a resource of exceptional value to the field going forward. Highlights include the construction of a mutant that lacks all b-1,6 glucan and the characterization of its cell wall composition and structure. Figure 5a is a feast for the eyes, showing that b-1,6 glucan is vital for the outer fibrillar layer of the cell wall. Also much appreciated was the summary figure, Figure 7, that presents the main findings in digestible form.

Strengths:

The work is highly significant for the fungal pathogen field especially, and more broadly for anyone studying fungi, antifungal drugs, or antifungal immune responses.
The manuscript is very readable, which is important because most readers will be cell wall nonspecialists.
The authors construct a key quadruple mutant, which is not trivial even with CRISPR methods, and validate it with a complemented strain. This aspect of the study sets the bar high.
The authors develop new and transferable methods for b-1,6 glucan analysis.

Weaknesses:

The one "famous" cell type that would have been interesting to include is the opaque cell. Please include it in the next paper!

https://doi.org/10.7554/eLife.100569.2.sa2

Reviewer #3 (Public review):

Summary:

The cell wall of human fungal pathogens, such as Candida albicans, is crucial for structural support and modulating the host immune response. Although extensively studied in yeasts and molds, the structural composition has largely focused on the structural glucan b,1,3-glucan and the surface exposed mannans, while the fibrillar component β-1,6-glucan, a significant component of the well wall, has been largely overlooked. This comprehensive biochemical and immunological study by a highly experienced cell wall group provides a strong case for the importance of β-1,6-glucan contributing critically to cell wall integrity, filamentous growth, and cell wall stability resulting from defects in mannan elongation. Additionally, β-1,6-glucan responds to environmental stimuli and stresses, playing a key role in wall remodeling and immune response modulation, making it a potential critical factor for host-pathogen interactions.

Strengths:

Overall, this study is well designed and executed. It provides the first comprehensive assessment of β-1,6-glucan as a dynamic, albeit underappreciated, molecule. The role of β-1,6-glucan genetics and biochemistry has been explored in molds like Aspergillus fumigatus, but this work shines important light on its role in Candida albicans. This is important work that is of value to Medical Mycology, since β-1,6-glucan plays more than just a structural role in the wall. It may serve as a PAMP and a potential modulator of host-pathogen interactions.

Weaknesses:

In keeping with an important role in immune recognition, it was suggested that the manuscript rigor would benefit from a more physiological evaluation ex vivo and preferably in vivo, assessment on stimulating the immune system within in the cell wall and not just as a purified component. This is a critical outcome measure for this study and gets squarely at its importance for host-pathogen interactions, especially in response to environmental stimuli and drug exposure. The authors addressed this issue contextually and indicate that it will require a more detailed immunologic evaluation but is not in keeping with the intent of this foundational study.

https://doi.org/10.7554/eLife.100569.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*

The fungal cell wall is a very important structure for the physiology of a fungus but also for the interaction of pathogenic fungi with the host. Although a lot of knowledge on the fungal cell wall has been gained, there is a lack of understanding of the meaning of ß-1,6-glucan in the cell wall. In the current manuscript, the authors studied in particular this carbohydrate in the important humanpathogenic fungus Candida albicans. The authors provide a comprehensive characterization of cell wall constituents under different environmental and physiological conditions, in particular of ß-1,6glucan. Also, β-1,6-glucan biosynthesis was found to be likely a compensatory reaction when mannan elongation was defective. The absence of β-1,6-glucan resulted in a significantly sick growth phenotype and complete cell wall reorganization. The manuscript contains a detailed analysis of the genetic and biochemical basis of ß-1,6-glucan biosynthesis which is apparently in many aspects similar to yeast. Finally, the authors provide some initial studies on the immune modulatory effects of ß-1,6-glucan.

Strengths:

The findings are very well documented, and the data are clear and obtained by sophisticated biochemical methods. It is impressive that the authors successfully optimized methods for the analyses and quantification of ß-1-6-glucan under different environmental conditions and in different mutant strains.

Weaknesses:

However, although already very interesting, at this stage there are some loose ends that need to be combined to strengthen the manuscript. For example, the immunological studies are rather preliminary and need at least some substantiation. Also, at this stage, the manuscript in some places remains a bit too descriptive and needs the elucidation of potential causalities.

Reviewer #2 (Public Review):

Summary:

The authors provide the first (to my knowledge) detailed characterization of cell wall b-1,6 glucan in the pathogen Candida albicans. The approaches range from biochemistry to genetics to immunology. The study provides fundamental information and will be a resource of exceptional value to the field going forward. Highlights include the construction of a mutant that lacks all b-1,6 glucan and the characterization of its cell wall composition and structure. Figure 5a is a feast for the eyes, showing that b-1,6 glucan is vital for the outer fibrillar layer of the cell wall. Also much appreciated was the summary figure, Figure 7, which presents the main findings in digestible form.

Strengths:

The work is highly significant for the fungal pathogen field especially, and more broadly for anyone studying fungi, antifungal drugs, or antifungal immune responses.

The manuscript is very readable, which is important because most readers will be cell wall nonspecialists.

The authors construct a key quadruple mutant, which is not trivial even with CRISPR methods, and validate it with a complemented strain. This aspect of the study sets the bar high. The authors develop new and transferable methods for b-1,6 glucan analysis.

Weaknesses:

The one "famous" cell type that would have been interesting to include is the opaque cell. This could be included in a future paper.

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The cell wall of human fungal pathogens, such as Candida albicans, is crucial for structural support and modulating the host immune response. Although extensively studied in yeasts and molds, the structural composition has largely focused on the structural glucan b,1,3-glucan and the surface exposed mannans, while the fibrillar component β-1,6-glucan, a significant component of the well wall, has been largely overlooked. This comprehensive biochemical and immunological study by a highly experienced cell wall group provides a strong case for the importance of β-1,6-glucan contributing critically to cell wall integrity, filamentous growth, and cell wall stability resulting from defects in mannan elongation. Additionally, β-1,6-glucan responds to environmental stimuli and stresses, playing a key role in wall remodeling and immune response modulation, making it a potential critical factor for host-pathogen interactions.

Strengths:

Overall, this study is well-designed and executed. It provides the first comprehensive assessment of β-1,6-glucan as a dynamic, albeit underappreciated, molecule. The role of β-1,6-glucan genetics and biochemistry has been explored in molds like Aspergillus fumigatus, but this work shines an important light on its role in Candida albicans. This is important work that is of value to Medical Mycology, since β-1,6-glucan plays more than just a structural role in the wall. It may serve as a PAMP and a potential modulator of host-pathogen interactions. In keeping with this important role, the manuscript rigor would benefit from a more physiological evaluation ex vivo and preferably in vivo, assessment on stimulating the immune system within in the cell wall and not just as a purified component. This is a critical outcome measure for this study and gets squarely at its importance for host-pathogen interactions, especially in response to environmental stimuli and drug exposure.

Response to reviewers (Public reviews):

We thank all the three reviewers for their opinion on our work on *Candida albicans* β-1,6-glucan, which highlights the importance of this cell wall component in the biology of fungi. Here are our responses to their comments for public reviews:

(1) Indeed, the data presented for immunological studies is preliminary. It has been acknowledged by the reviewers that our analysis providing insights into the biosynthetic pathways involved in comprehensive in dealing with organization and dynamics of the β-1,6-glucan polymer in relation with other cell wall components and environmental conditions (temperature, stress, nutrient availability, etc.). However, we anticipated that there would be immediate curiosity as to what the immunological contribution of β-1,6 glucan and we therefore felt we needed to initiative these studies and include them. We therefore performed immunological studies to assess whether β-1,6-glucans act as a pathogen-associated molecular pattern (PAMP), and if so, what its immunostimulatory potential is. Our data clearly suggest that β-1,6-glucan is a PAMP, and consequently lead to several questions: (a) what are the host immune receptors involved in the recognition of this polysaccharide, and thereby the downstream signaling pathways, (b) how is β-1,6-glucan differentially recognized by the host when *C. albicans* switches from a commensal to an opportunistic pathogen, and (c) how does the host environment impact the exposure of this polysaccharide on the fungal surface. We believe addressing these questions is beyond the scope of the present manuscript and aim to present new data in future manuscript. Nonetheless, in the revised manuscript,

suggest approaches that we can take to identify the receptor that could be involved in the recognition of β-1,6-glucan. Moreover, we have modified the discussion presenting it based on the data rather than being descriptive.

(2) It will be interesting to assess the organization of β-1,6-glucan and other cell wall components in the opaque cells. It is documented that the opaque cells are induced at acidic pH and in the presence of *N*-acetylglucosamine and CO2. Our data shows that pH has an impact on β-1,6-glucan, which suggests that there will be differential organization of this polysaccharide in the cell wall of opaque cells. As suggested by the reviewer, we will include analysis of opaque cells (and other *C. albicans* cell types) in future studies.

With the exception of these major new avenues for this research, our revision can address each of the comments provided by the reviewers.

> ***Recommendations for the authors***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Although the study is very interesting, there are some loose ends that need to be combined to strengthen the manuscript. For example, the immunological studies are rather preliminary and need at least some substantiation. Also, at this stage, the manuscript in some places remains a bit too descriptive and needs the elucidation of potential causalities.*
>
> *Specifically:*
>
> *(1) As you showed, defects in chitin content led to a decrease in the cross-linking of β-glucans in the inner wall that corresponded to the effect of nikkomycin-treated C. albicans phenotype; conversely, an increase in chitin content led to more cross-linking of β-glucans as observed in the FKS1 mutant or in the presence of caspofungin. What is the mechanistic reason for these observations?*

On one hand, yeast cell wall chitin occurs in three forms: free and covalently linked to β-1,3-glucan or β-1,6-glucan; crosslinked β-glucan-chitin forms core fibrillar structure resistant to alkali. A decrease in the chitin content, therefore, affect β-glucan-chitin crosslinking thereby making β-glucan alkali-soluble. On the other hand, a decrease in the β-glucan content, as in *FKS1* mutant or upon caspofungin treatment, results in increased cell wall chitin and β-glucan-chitin contents. A decrease in the β-1,3-glucan biosynthesis is associated with upregulation of *CRH1* involved in the β-glucan-chitin crosslinking, which explains an increased β-glucan-chitin content in the *FKS1* mutant or upon caspofungin treatment. We have included in this discussion in the revised manuscript (p14, lines 2-10).

> *(2) The β-1,6-glucan biosynthesis is stimulated via a compensatory pathway when there is a defect in O- and N-linked cell wall mannan biosynthesis. Why? causality? Hypothesis?*

Two phenomena were observed related to β-1,6-glucan and mannan biosynthesis: 1) a defect in the elongation of N-mannan led to an increase in the β-1,6-glucan content; 2) a defect of O-mannan elongation resulted in the reduce size of β-1,6-glucan chains, however, increased their branching. These observations of our study suggest a global rescue program of the cell wall damage that could occur due to defect in one of the cell wall contents. We have discussed this in the revised manuscript (p14, last paragraph, p15 first paragraph). Moreover, β-1,3-glucan and chitin are synthesized by respective membrane bound synthases, and a defect in of their synthesis is compensated by the other. In line, although need to be validated for β-1,6-glucan, biosynthesis of mannan and β-1,6-glucan seem to initiate intracellularly. Therefore, possibility is that the defective mannan biosynthesis could be compensated by β-1,6-glucan biosynthesis, but need to be further validated experimentally.

> *(3) You showed that the removal of β-1,6-glucan by periodate oxidation (AI-OxP) led to a significant decrease in the IL-8, IL-6, IL-1β, TNF-α, C5a, and IL-10 released, suggesting that their stimulation was in part β-1,6-glucan dependent. What is the consequence of the stimulation, e.g. better phagocytosis, etc.? This needs some more experiments, otherwise the data is purely descriptive, as the conclusion. Also, what do you want to show with the activation of the complement system? Is ß1,6-glucan detected by complement receptors? I think this is really a loose end. I think it is necessary to provide more data on this observation, which I think lacks control with serum lacking complement, this should then be moved to the main manuscript.*

In this study, our aim was to assess whether β-1,6-glucan acts as a pathogen-associated molecular pattern (PAMP) of *C. albicans*, and if yes, what is its immunostimulatory capacity/potential. Our data confirms that, indeed, β-1,6-glucan acts as a PAMP, and its removal significantly reduces the immunostimulatory capacity of the fibrillar core structure of the *C. albicans* cell wall. On the other hand, data provided in the revised manuscript (see updated Figure S14, discussion p13 lines 16-21) indicate that the human serum factors significantly enhance the immunostimulatory capacity of β1,6-glucan and that β-1,6-glucan interacts with the complement component C3b. However, addressing the role of β-1,6-glucan in phagocytosis using β-1,6-glucan deletion mutant will not be possible as the cell wall of this mutant is modified, and β-1,6-glucan is not the only cell wall component interacting with C3b. Alternate is to coat β-1,6-glucan on beads and use to study phagocytosis and identify immune receptors; however, these are beyond the scope of our present study/focus.

> *(4) Also, you suggested that β-1,6-glucan and β-1,3-glucan stimulate innate immune cells in distinct ways. Please provide more data on this interesting suggestion. You can block the dectin-1 receptor for example or use dectin-1 deficient macrophages from mice. The part on the immune stimulation needs to be optimized.*

Stimulation of immune cells by pustulan (insoluble linear β-1,6-glucan) via a dectin-1independent pathway has been described previously (PMIDs: 18005717, 16371356) as discussed in the manuscript. Our preliminary data indicate that dectin-1 blocking on immune cells (using antidectin-1 antibodies) has no effect on the immunostimulatory potential of β-1,6-glucan, unlike AI and AI-OxP that showed significantly reduced cytokine secretion by the immune cells upon dectin-1 blocking. Deciphering the β-1,6-glucan recognition and its immunomodulatory pathways are underway, and will be the subject of our future study/manuscript.

> *(5) β-1,6-glucan and mannan productions are coupled. What is the hypothesis? Is it due to the necessity of mannan residues in ß-1,6-glucan biosynthesis enzymes from the ER? Can that be experimentally proven?*

β-1,6-glucan and mannan synthesis should be coupled in two ways. First, as mentioned above (Response 2), defects in mannan elongation led to an alteration of β-1,6-glucan production. Second, early steps of N-glycosylation led to a strong reduction of β-1,6-glucan size and its cell wall content. However, we do not believe that the synthesis of N-glycan is required for the synthesis of an acceptor essential to β-1,6-glucan synthesis. Defect in N-mannan elongation led to a global cell wall remodeling as described above. Kre5, Rot2 and Cwh41 are part of the calnexin cycle involved in the control of N-glycoprotein folding in the ER, suggesting that some protein directly involved in the β-1,6-glucan synthesis required a folding quality control to be active. We modified our discussion, accordingly, highlighting these points (p14, last paragraph, p15 second paragraph).

> *(6) As PHR1 and PHR2 genes are strongly regulated by external pH, the compensatory differences described may be explained by pH-dependent regulation of β-1,6-glucan synthesis.' Please check. Also, could the pH regulation form the basis of e.g. differences you found for ß-1,6-glucan under different environmental conditions, i.e., growth on different carbon sources leads to different external pH values, as shown for many fungi?*

We agree that environmental pH is dependent on carbon source and pH varies during growth curve. To test the effect of pH we buffered the medium with 100 mM MOPS or MES. Clearly, Fig. 2 and S1 show that the pH has an effect on the cell wall composition and polymer exposure as previously described (PMID: 28542528). Here, we show that pH has an impact on the β-1,6-glucan size as well as its branching. However, in buffered medium, addition of organic acid (such as acetate, propionate, butyrate or lactate) had an impact on cell wall composition, showing that not only pH has an effect on cell wall composition. About *phr1*Δ/Δ and *phr2*Δ/Δ mutants, we believe that the difference in the cell wall composition observed between mutants is mainly due to the pH-dependent regulation, which we indicated in the discussion (p14, end of first paragraph).

> *Minor:*
>
> *(1) In Figure 7B: dynamism should be replaced by dynamic and in term is rather in terms.*

Modified as suggested.

> *(2) Replace molecular size with molecular mass when you give daltons.*

Molecular size has been replaced by molecular weight, when presented as daltons.

> *(3) Page 7: for explanation, please add that nikkomycin is a chitin biosynthesis inhibitor.*

As suggested, explained that nikkomycin is a chitin biosynthesis inhibitor.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) I wondered if the increased chitin content of hyphae might reflect growth on the precursor GlcNAc. Have you tested hyphae that are induced in other ways? (2) Related to point 1, did you look at the relative abundance of yeast vs hyphae in the preparation? I wonder if yeast contamination might have reduced the extent of the composition changes observed.*

We used GlcNAc as hyphae inducer as: 1) in presence of GlcNAc, hyphae are produced without any yeast contamination; in this condition, we observed an increase in the chitin content, as described, in hyphae (PMID: 16423067); 2) we excluded using of serum, another condition inducing hyphal formation, as we could not control serum factors that may impact cell wall composition. We now indicate in the methods section that hyphae induced by GlcNAc were not contaminated by yeast (p17, line 3).

> *(3) I recommend rephrasing the first sentence of the Figure 2 legend: "Cells were grown in liquid SD medium at 37oC at exponential phase under different growth conditions." The conditions varied extensively - stationary is not exponential; biofilm is probably not exponential. Also, the "D" in "SD" stands for dextrose, and the carbon source varied a good deal. Perhaps you could say: "Cells were grown in liquid synthetic medium at 37oC under different growth conditions, as specified in Methods."*

Sentences have been rephrased.

> *(4) Figure 7b has a typo: "dependant" for "dependent".*

Typo-error has been corrected.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *To explore the biochemical composition of the cell wall, the authors fractionated the wall component into three categories based on polymer properties and reticulations: sodium-dodecyl-sulphate-βmercaptoethanol (SDS-β-ME) extract, alkali-insoluble (AI), and alkali-soluble (AS) fractions, and they developed several independent methods to distinguish between β-1,3-glucans and β-1,6-glucans. The composition and surface exposure of fungal cell wall polymers is known to depend on environmental growth conditions. It was shown that the cell wall of C. albicans hyphae increased chitin content (10% vs. 3%) and decreased β-1,6-glucan (18% vs. 23%) and mannan (13% vs. 20%) compared to the yeast form, and the reduced β-1,6-glucan content was associated with a smaller β1,6-glucan size (43 vs. 58 kDa), suggesting that both the content and structure of β-1,6-glucan are regulated during growth and cellular morphogenesis. Similar behavior was observed when exposing cells to acid and neutral medium pH. The most significant cell wall alteration occurred in a lactatecontaining medium, which led to a sharp reduction in structural core polysaccharides: chitin (-43%), β-1,3-glucan (-48%), and β-1,6-glucan (-72%). This reduction aligns with the previously observed decreases in inner cell wall layer thickness. As expected, the authors found that modulating chitin content genetically (chs3Δ/Δ knockout mutant) led to an increase of both β-1,3-glucan and β-1,6glucan. An increase in chitin content following genetic alteration of FKS genes impacting glucan synthase or after exposure to the echinocandin caspofungin led to enhanced cross-linking of βglucans. A slight increase in the β-1,3-glucan branching was also observed in the mnt1/mnt2Δ/Δ double mutant, suggesting that β-1,6-glucan and mannan synthesis may be coupled.*
>
> *- This effect is not that pronounced, and the relationship appears somewhat overstated and may reflect an indirect interaction. The authors should address accordingly.*

We agree that this sentence was overstated. To make it clearer and less pronounced, we divided this sentence into to two with less pronounced statements (p8, line 34).

> *The genetics of β-1,6-glucan biosynthesis appear complex and a figure describing putative roles for specific genes would be beneficial. For example, KRE6 is a glucosyl hydrolase required for beta1,6-glucan biosynthesis.*
>
> *- It would be valuable to better understand the overall biosynthetic process. Please elaborate more in a figure.*

Although proteins/enzymatic activities directly involved in the β-1,6-glucan biosynthesis have not yet been identified, as suggested by this reviewer, we included a schematic representation of this process based on our hypothesis (Figure S15, and p15 lines 17-22 in revised manuscript), indicating the possible involvement of Kre6p.

> *The deletion of KRE6 homologs, essential for β-1,6-glucan biosynthesis, resulted in the absence of β-1,6-glucan production, and significant structural alterations of the cell wall. This result nicely confirms the important role of β-1,6-glucan in regulating cell wall homeostasis. The absence of β1,6-glucan was associated with increased (mutant v. WT) chitin content (9.5% vs. 2.5%) and highly branched β- β-1,6-glucan 1,3-glucan (48% vs. 20%). TEM ultrastructure studies nicely showed the change in cell wall overall architecture. From a drug discovery perspective, since the blockade of β1,6-glucan did*

> *not block growth, it may have more value as a potential virulence target. This would be valuable but needs to be assessed in animal model challenge competition experiments.*
>
> *- The authors may want to elaborate more.*

We agree and modified “antifungal target” as “potential virulence target”.

> *It is well known that β-1,3-glucan, mannan, and chitin function serve as PAMPs, which induce immune responses. The role of β-1,6-glucan as a PAMP is not well understood, and the authors provide evidence that different cell wall extracted fractions with enriched constituents induce immune responses invoking cytokines, chemokines, and acute phase proteins, as well as the complement system. While this data clearly shows that β-1,6-glucan is immunologically active and potentially important for host-pathogen interactions, the analysis is preliminary and falls short of making this case.*
>
> *- This is a critical point in getting at the potential host signaling of β-1,6-glucan contained in the cell wall or shed by the cell (is this known?)*
>
> *- This analysis would be bolstered significantly by examining stimulation relative to other cell wall components, and most importantly, whole cell modulation of β-1,6-glucan exposure for immune presentation, and not just unnatural concentrated extracts. This can be readily accomplished with the various mutants in hand, as well as after exposure to various antifungal agents echinocandins and nikkomycins) (see Hohl et al. 2008 JID). Additional validation would benefit from animal model studies to examine in vivo immune modulation.*

We agree with the reviewer. However, the main focus of our present work was to study the organization and dynamics of *C. albicans* cell wall β-1,6-glucan, and to explore its possible role as pathogen-associated molecular pattern (PAMP). Our study indicates that, indeed, β-1,6-glucan acts as a PAMP with immunostimulatory potential. As pointed by this reviewer, and similar to β-1,3glucans, the exposure of β-1,6-glucan is probably a key point in immune response. However, this investigation beyond the scope of this study, underway and will be presented in our future work.

> *- The Discussion would also benefit from an analysis of how β-1,6-glucan in Aspergillus fumigatus, which was largely elucidated by the same primary authors.*

To our knowledge, β-1,6-glucan has never been identified, either by chemical analysis (PMID: 10869365; PMID: 36836270) or solid-state NMR (PMID: 34732740), in the cell wall of *A.*

fumigatus, although a homolog of *KRE6* is present in *A. fumigatus* but with unknown function.

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