The yeast Saccharomyces cerevisiae cell wall: Molecular architecture, regulatory pathways and remodelling mechanisms in response to environmental conditions, and biotechnological values

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ABSTRACT

Yeast cells are surrounded by a thick cell wall which ensures their shape, their integrity and constitutes the first interface with the environment. The yeast cell wall consists of a complex structure of polysaccharides chains, mostly β-glucans and a small amount of chitin, interconnected to mannoproteins. Cell division, developmental programs and response to environmental changes lead to extensive cell wall remodelling. These alterations are controlled by complex signalling pathways involving stress sensors, MAP kinases and several transcription factors. Understanding the molecular mechanisms underlying yeast cell wall assembly represents a great interest for biotechnological processes including research of new antifungal agents and new cell targets.

INTRODUCTION

The yeast cell wall is the main determinant of cellular strength, and plays an important role in cell morphogenesis and cell growth. It is the first cellular structure in direct contact with the surroundings. Under a normal growth situation, the cell wall amounts to about 20 % of the cell dry mass. The yeast cells have a very high turgor pressure, and a minor chink in the cell wall can lead to bursting and death. Moreover, the cell wall is not a rigid structure as it endures all the changes that the cell undergoes during division, morphogenesis and differentiation. To ensure a continuous integrity of the wall in accordance with its plasticity, elaborated mechanisms must be operating, which need to be strictly coordinated with those governing the cell cycle progression. This review will focus on recent data on the cell wall biogenesis and remodelling in response to environmental stress and in relation to cell growth. Emphasis on the reasons to study yeast cell wall will be depicted through the biotechnological values of the cell wall components and as an attractive antifungal target. The reader wishing more detailed information about the molecular organisation of the yeast wall, or on specific metabolic or mechanistic issues related to cell wall synthesis may consult excellent reviews that have appeared very recently [1-6].

CELL WALL COMPOSITION AND BIOGENESIS

The cell wall of *Saccharomyces cerevisiae* is organized into two layers that are made up of four classes of covalent cross-linked macromolecules: β1,3-glucan, β1,6-glucan, chitin and cell wall mannoproteins (CWPs). Electron-microscopic studies have shown that the mannoproteins form a fibrillar outer layer extending radially from the inner skeletal layer, which is formed by the polysaccharide fraction of the cell wall [7]. The architecture of the cell wall from the yeast *S. cerevisiae* is outlined in Figure 1.

β 1,3-glucans: structure, enzymology and regulation

The β1,3-glucans, the most prominent carbohydrates of the cell wall, account for about 50% of the cell wall dry mass. They form a fibrillar structure, composed of three helically entwined linear chains of about 1500 β(1→3) linked glucose units [8]. This structure provides the rigidity and integrity of the cell wall, and determines the cell shape. The β1,3-glucans are produced from UDP-glucose by the membrane-bound β1,3-glucan synthase (GS) encoded by two homologs genes, *FKS1* and *FKS2* [9;10].

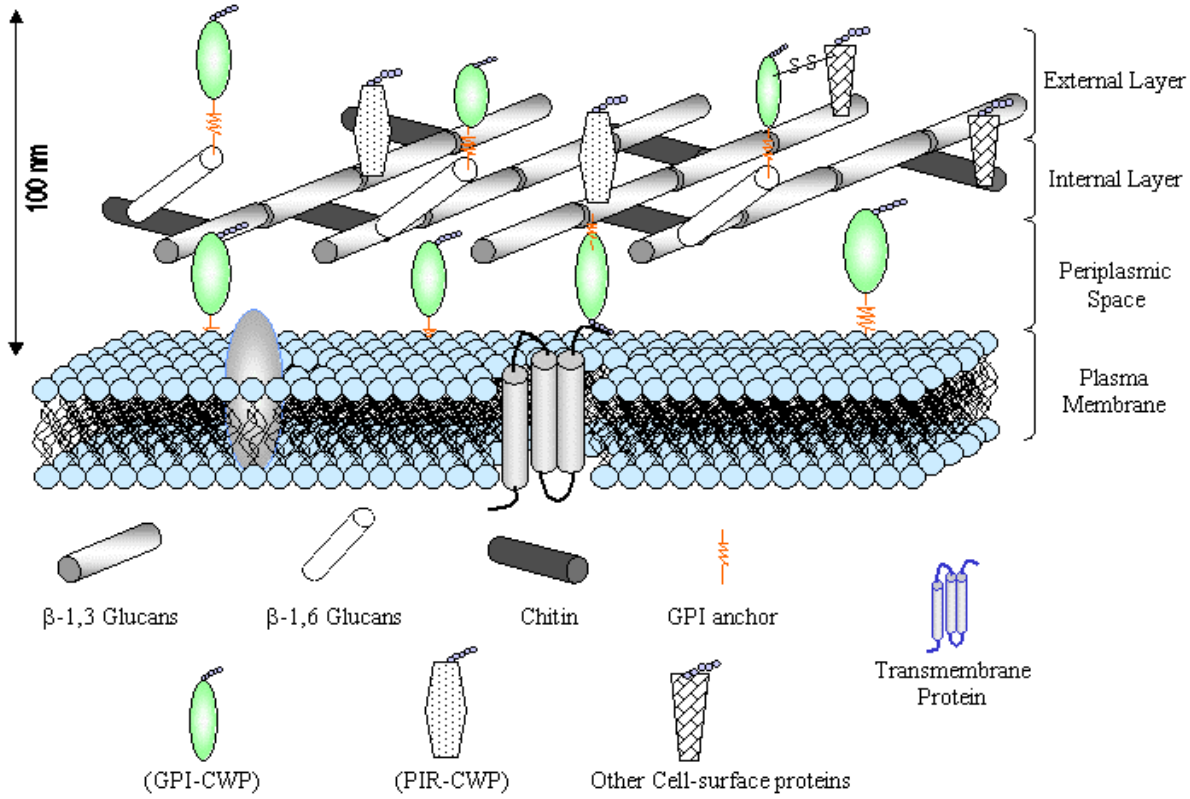

Figure 1: Molecular model of the *Saccharomyces cerevisiae* cell wall.
Yeast cell wall consists of a 100 nm thick extra-cellular matrix. The internal skeletal layer is made of β1,3-glucans that form a three dimensional network surrounding the entire cell and strengthened by chitin fibers. β 1,3-glucans are branched with β1,6-glucans side chains which also interact with chitin and function as a flexible tether for GPI-anchored mannoproteins (GPI-CWPs). A second class of mannoproteins (PIR-CWP) are directly linked to β1,3- glucans. Other cell-surface proteins are attached to cell wall either by disulphide bridges to other CWPs, or by non specific binding.

The expression of *FKS1* is more abundant during vegetative growth and is in part cell cycle regulated, while *FKS2* is transcribed under stress conditions or in response to cell wall defects such as in a *fks1* mutant [10-12]. Thus, only the double knock out of *FKS1* and *FKS2* is lethal. While Fks1 and Fks2 are suspected to correspond to the catalytic subunit of GS [13], it is however clear that the GS activity requires the presence of a 26 kDa GTP binding protein encoded by *RHO1* [14]. This small G-protein is loosely attached to the plasma membrane through prenylation at the C-terminus. Rho1 acts as a 'switch' of the GS activity due the conversion between an inactive GDP-bound to an active GTP-bound form catalysed by the guanine exchange factor Rom1 and Rom2 [15]. Conversely, GTPase-activating proteins, encoded by *BEM2* and *SAC7*, switch off the GS activity by converting GTP-bound active Rho1 to the inactive GDP-bound form[16;17]. Very recently, it was found that GS was non-competitively inhibited by a physiological concentration of phytosphingosine, an intermediate of the sphingolipid biosynthesis that is located in the endoplasmic reticulum [18]. This inhibition was supposed to have a regulatory role in the interaction between Fks1 and Rho1. To conclude, many questions are not yet solved on the structure-function, and regulation, of the β1,3-glucan synthase isoforms in yeast, which therefore precludes decisive antimicrobial development towards this potential target.

β1,6-glucans: structure, enzymology and regulation

The structure of β-glucans also comprises a minor amount (about 5 %) of β1,6-glucan, a polymer of *ca* 100-350 β (1→6) linked glucose units. The biosynthesis of this polymer, which likely arises from UDP-glucose as the glucosyl donor, has been mainly studied by a genetic approach (see [4], for an extensive review), and a 'β1,6-glucan synthase' enzyme has not been discovered yet. Nonetheless, this polymer is an essential fungal-specific component of the cell wall that interconnects all other wall components into a lattice (see Figure 1 and 2). The genetic approach which took the advantage that cells devoid of β1,6-glucan were highly resistant to K1 Killer toxin (called *kre* mutants for Killer REsistant) has elucidated only part of the biosynthetic pathway of this polymer. The biosynthesis is initiated in the endoplasmic reticulum, continues in the Golgi apparatus and ends up at the cell surface. However, Klis group [19] failed to identify intracellular material that reacts with specific β1,6-glucan antibodies, even in mutants of the secretory pathway. Thus, the complete metabolic scheme leading to the synthesis of β1,6-glucans, as well as the biochemical activity corresponding to most of the isolated *KRE* genes remains to be elucidated.

Chitin: structure, enzymology and regulation

Chitin is an essential component of the yeast cell wall even though it amounts to only 1-2% of the cell wall dry mass in a wild type strain [20]. It is a linear homopolymer composed of *ca* 120 *N*-acetylglucosamine residues that are linked by β(1→4) bonds. Chitin structure consists of antiparallel hydrogen-bonded chains called microfibrils that can retain the dye Calcofluor White [21-23]. In *S. cerevisiae* and in the pathogenic yeast *Candida albicans*, it has been shown that chitin is attached covalently to β 1,3 and β1,6-glucans [24-27]. Since β 1,6-glucans are in turn attached to the glycosylphosphatidylinositol (GPI)-containing cell wall proteins, all components are attached directly or indirectly to chitin. In *S. cerevisiae*, the formation of chitin is taken over by three distinct chitin synthases, encoded by *CHS1*, *CHS2* and *CHS3* [28-32]. They are integral membrane proteins with the catalytic domain located at the cytoplasmic face. Localisation of chitin synthases has been also reported in vesicles named chitosomes [33;34]. Chitin synthases are all stimulated by acetylglucosamine and feedback inhibited by UDP. The activity of the three chitin synthases can be distinguished by their differential sensitivity to Mg^{2+}, Co^{2+} or Mn^{2+} [35] and, in the case of Chs1 and Chs2, by a '*in vitro* activation' after mild treatment with trypsin, suggesting that the two latter enzymes occur naturally in a zymogenic state [28]. The existence of three distinct chitin synthases raises questions about their specific roles and regulation mechanism in yeast. These questions have been addressed at the genetic and biochemical levels. As it was shown that chitin synthesis is a cell cycle regulated process, the action of each of the chitin synthases can be assigned to specific steps during this process. Chs1 is a repair enzyme that synthesises chitin in response to an acidification event following separation of mother and daughter cells [36]. Because the *in vitro* activity of Chs1 cannot be detected without proteolytic treatment, it is thought that Chs1 is tightly controlled *'in vivo'* by a proteolytic system that has not been identified yet. Chs2 is localised to the mother - bud septum and is responsible for primary septum formation between mother and daughter cells [37;38]. Chs3 is the most important enzyme catalysing *ca* 90 % of the chitin content of the cells. This enzyme is spatially and temporarily controlled by a number of regulatory proteins encoded by *CHS4*, *CHS5*, *CHS6* and *CHS7* [39-44]. The interaction of Chs3 with Chs4 that in turn

interacts with one of the septin protein, Bni4 (Bud-Neck-Involved) may account for the targeting of Chs3 to a ring at the mother-bud neck. This interaction could explain that Chs3 is no longer localised at this site in a *bni4*Δ or *chs4*Δ mutant [39;45]. The Chs5 and Chs6 proteins have also a role of targeting the Chs3 to polarized growth sites [41-43]. Chs7 is a membrane protein that is required for the export of Chs3 from the ER, which together with Chs4 determines the full Chs3 activity *in vivo* [39]. The levels and localisation of the three chitin synthases have been monitored through the cell cycle. Chs1 and Chs3 are synthesized constitutively, and localised in plasma membrane and/or in chitosomes, with a probably cell-cycle dependent trafficking of chitin synthases between these two cellular structures [34;46]. This cycling mechanism constitutes a mean to maintain a constant pool of enzymes for chitin synthesis. Chs2 does not follow this pathway and probably processes via the secretory pathway, where it is packaged and transported to the plasma membrane at the site of septum formation. The enzyme is then internalised and degraded when it is no longer needed [34].

The genome of *S. cerevisiae* contains two genes *CTS1* and *CTS2* encoding endochitinases, which cleave off chitin into *N*-acetylglucosamine units. Cts1 is present in vegetative cells as a protein non-covalently bound to cell wall [47]. The main function of this hydrolase is to dissolve the chitinous primary septum that is synthesized by Chs2 during cell separation. Since excessive activity can be lethal for the cell, the Cts1 is supposed to be tightly controlled by both transcriptional and post-translational mechanisms [48-50]. The *CTS2* gene is induced during sporulation, but the role of *CTS2* product during this process remains unknown.

Mannoproteins: structure, enzymology and regulation

The outer layer of the yeast wall is made up of proteins that are bound via a serine, threonine or an asparagine residue to a polysaccharide complex of 150 or more *D*-mannose units (the so-called mannan). Early chemical studies have shown that the mannans are composed of an $\alpha(1\rightarrow6)$ linked backbone of mannoses to which are attached short side chains of mannoses linked by $\alpha(1\rightarrow2)$ and $\alpha(1\rightarrow3)$ bonds. The polysaccharide structure can reach up to 50 % of cell wall dry mass. The biosynthetic pathway of mannans starts in the cytosol by the isomerisation of fructose-6*P* into mannose-6*P* catalysed by the phosphomannose isomerase (Pmi1), which is then epimerised into mannose-1*P*. The GDP-mannose is formed from mannose-1*P* and GTP by a GDP-mannose pyrophosporylase encoded by the essential gene *PSA1* [51]. A dolichol-phosphate synthase, localised at the cytosolic face of the ER and encoded by the essential gene *DPM1*, transfers the mannose from GDP-mannose to Dolichol-phosphate to form Dol-*P*-mannose. This molecule is a key intermediate in three protein glycosylation processes, namely the *N*-glycosylation, *O*-glycosylation and glycosylphosphatidyl-inositol (GPI) membrane anchoring [52]. These reactions all occur in the rough endoplasmic reticulum.

N-glycosylation involves a set of glycosyltransferases encoded by *ALG1* to *ALG10* genes which produce the oligosaccharide precursor $GlcNac_2Man_9Glc_3$ on a polyisoprenoid carrier lipid Dol-*PP*. The last step in the ER is the transfer of the oligosaccharide to the amide group of an asparagine residue of the protein in the tripeptide ‘sequon’ Asn-X-Ser/Thr, where X is any amino acid except proline. This reaction is catalysed by the oligosaccharide transferase (OTase) complex composed of eight proteins, all of which are encoded by essential genes [53;54]. Maturation of glycolysated protein

occurs in the Golgi apparatus by successive addition of mannose units from GDP-mannose catalysed by specific α-mannosyltransferases.

The *O*-mannosylation is initiated in the ER by transferring a mannose from Dol-*P*-mannose to a Ser or Thr residue of the protein. This reaction is carried out by a Dol-*P* Man::protein *O*-mannosyltransferase encoded by one of the seven *PMT* genes [55;56]. Only deletion of three of them (*PMT1, 2* and *4* or *PMT2, 3* and *4*) leads to inviability, leaving the other four with no clear function yet. Elongation of *O*-linked chains, *i.e*. addition of 2 to 4 more mannoses relies on GDP-mannose as mannosyl donor, and these reactions that also occur in the Golgi apparatus are catalysed by α-mannosyltransferase [57;58]. It can be noticed that most glycoproteins of *S. cerevisiae* are both *N* and *O*-glycosylated.

Once the mannoproteins have reached the outer cell surface, they can be retained to β1,6-glucan through a remnant of the GPI anchor which they have received during maturation in the endoplasmic reticulum (the so-called GPI-CWPs). Lack of this GPI-anchor results in secretion of the CWPs in the medium [26;59;60]. A second class of cell wall mannoproteins characterized by Protein Internal Repeat regions (PIR-CWPs) are directly linked to β1,3-glucan and exhibit not obvious function [61;62]. A last category are cell-surface proteins attached to cell wall either by disulphide bridges to other CWPs, or by 'affinity' or non specific binding as it is the case for many glycolytic enzymes [63-65].

THE MODULAR STRUCTURE OF THE CELL WALL

The structural components described above are not simply juxtaposed. They are rather assembled to each other by covalent linkages, which generate a modular architecture of the yeast cell wall (see Figure 2). The first fully characterized linkage was a β(1→4) link between the reducing terminal GlcNac residue of a chitin chain and the non reducing terminal glucose of a β1,3-glucan chain [66]. Covalent association between mannoproteins, β1,6-glucan and β1,3-glucan have been identified later on [24;26;67;68]. Thus, the cell wall modules, i.e. CWP → β1,6-glucan ← chitin and CWP → β1,6-glucan → β1,3-glucan ← chitin involve glycosidic attachments between β-glucans and chitin. GPI-CWPs mannoproteins are linked to β1,6-polysaccharide via a processed form of GPI anchors. The remnant structure of the GPI anchor of CWPs to which β1,6-glucan is attached harbours the ethanolamine-PO_4-Man_5 structure [69]. The PIR-CWPs are on the other hand directly linked to the β1,3 glucan through an alkali-labile linkage of yet unidentified nature [3;61]. A model of the yeast cell wall building block is shown in Figure 2.

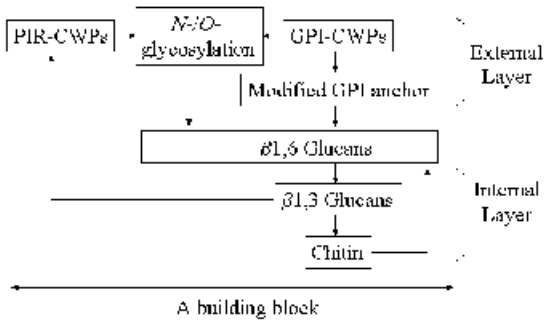

Figure 2: Typical building blocks of the yeast cell wall. Relationships among components of a cell wall module (or building block) are schematically depicted on this figure. See in the text for further details.

Apparently not all modules actually contain chitin, which accounts for the existence of two chemical fractions of β-glucan : a chitin-free alkali-soluble fraction and a chitin-enriched alkali-insoluble one. The increased amount of the latter fraction is indicative of a chitin enrichment of the cell wall [20;26;70]. The different modules are interconnected by noncovalent interactions in the β-glucan-chitin layer

and by covalent cross-links in the mannoprotein layer including disulfite bonds between mannoproteins [1;12;71] and perhaps also through other mannoprotein-glucan links not yet characterized [26].

Although the chemical bonds between the different cell wall components have been described in details, the enzymes involved in the generation of these links are largely unknown. These are enzymes involved in the cleavage of the GPI-anchor of the GPI-proteins and the subsequent attachment to β1,6-glucans, as well as enzymes involved in the coupling between β-glucan and chitin. Yeast possesses several exo-glucanases that catalyze *in vitro* the hydrolysis of linkages at the non-reducing ends of β1,3-glucans, and endo-glucanases activities which cleave within the chains [20;72]. However, some of the identified β-glucanases may also carry out glycosyl transferase activity. This is the case for Bgl2 which was originally characterized as an endo-β1,3-glucanase [73;74], and latter on found to display also a glycosyl transferase activity [75]. Apparently, a low water environment, which is likewise the situation existing in the cell wall, favours this transferase activity [75]. Another example of β1,3-glucanosyltransferase has been decrypted recently by investigating the kinetic properties of Gas1, a highly glycosylated protein which belongs to a family of related yeast and fungal proteins attached to cell membrane by a GPI-anchor [2]. Gas1 plays a major role in cell wall assembly as mutants defective in this protein display aberrant phenotypes, such as very slow-growth, clumpiness, high thermotolerance, hypersensitivity to cell-wall perturbing drugs [12;76], reduction of incorporation in cell wall of β1,3-glucan and more abundant release of β1,6-glucosylated GPI-mannoproteins in the growth medium [77]. These pleiotropic defects can be a consequence of the inability of the Gas1 enzyme to create anchoring sites for mannoproteins on glucan chains by its transferase activity [78] Many other genes, as for instance the *SCW* gene family [47;79] or the *CRH1* and *CRH2* [80] that encode proteins with putative glycosyl transferase activity may also be implicated in cross-linking of the cell wall components. These assembly enzymes are interesting candidates as antifungal targets since their inactivation should weaken cell wall structure and eventually inhibit growth. This challenge is being raised by a European project named "EUROCELLWALL" under the European Commission Framework V (see http://www.insa-tlse.fr/gba/Recherche /Equipes/Eucariote/fr).

COORDINATION OF CELL WALL INTEGRITY AND CELL GROWTH

During cell growth and development, the cell wall undergoes extensive remodelling and modifications in its structure and composition. Regulation of cell wall assembly must be coordinated with cell cycle control during vegetative growth, as well as during the different developmental programs such as mating, sporulation and formation of pseudo-hyphae. Since these questions have been recently reviewed [5;81], we will mainly focus this part on recent data about mechanisms that coordinate cell wall integrity to cell proliferation.

The cell wall integrity is mainly under the control of the Pkc1-MAP kinase pathway

Cells sense and respond to environmental constraints via signalling pathways. As shown in Figure 3, five signalling pathways based on a module of three protein kinases highly conserved among eucaryotes and which culminate in the activation of a mitogen-activated protein kinase (MAPK) have been analysed in great details in the yeast *S. cerevisiae* [81-83]. It is particularly important to notice that

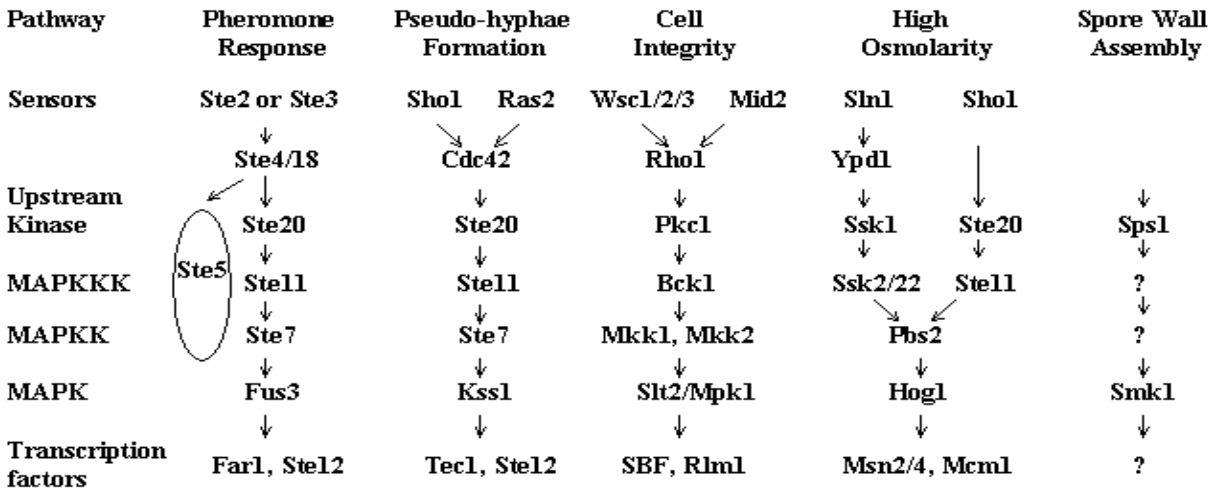

Figure 3: MAP kinase cascades in *Saccharomyces cerevisiae*.
Five different signalling pathways of *S. cerevisiae* involve MAPK modules, highly conserved among eucaryotes, which consist of a MAPKKK (or MEKK), a MAPKK (or MEK), and a MAPK (ERK). The receptors which activate the MAPK modules in response to extracellular signals (Inputs) are specific for each of these pathways. The substrates of the activated MAPK (Outputs), among which many are transcription factors, are also specific for each pathway and induce specific cellular responses such as altered patterns of gene expression and protein activity. Question marks indicate that no protein has been identified yet for that step in the cascade.

some of the signalling pathways can share common elements. This partial overlapping actually does not interfere with the specificity of the response, which depends largely on the upstream (sensors) and downstream (transcription factors) elements that are specific to each pathway. The sharing of partners likely illustrates a cross talk between the various pathways that is necessary to produce a coordinated cellular response.

The MAPK cascade dependent on the protein kinase C encoded by *PKC1* is considered as the main pathway controlling cell wall integrity. The *PKC1* gene of *S. cerevisiae* was isolated as homologous of the mammalian *PKC* gene [84]. Pkc1 protein kinase is essential for the formation of an osmotically stable cell wall since loss of its function results in cell lysis at all temperatures, a phenotype that can be partially remediable by osmotic stabilizators, such as sorbitol 1M [84;85]. The signalling pathway is constituted of two branches that diverge downstream of the Pkc1. One of them is a linear pathway consisting of the sequentially activated protein kinases Bck1, the redundant Mkk1/Mkk2 and the Slt2/Mpk1 MAPK, which ultimately activates by phosphorylation transcription factors including Rlm1 and SBF complex [86;87]. The evidence that there is another branch arose from the finding that phenotypes of a *pkc1* null mutant are stronger than those of mutants downstream of the cascade [88]. However, the components of this second branch have not been completely worked out (see below). The Pkc1- MAP kinase pathway is activated by cell wall stress such as heat, hypo-osmotic conditions, mutations of cell wall structural genes, cell-wall perturbing drugs like Calcofluor White, Congo Red, caffeine, SDS, zymolyase [12;89-91]. This activation is transmitted through plasma membrane sensors including Hcs77 /Slg1/Wsc1, Wsc2, Wsc3, Wsc4 and Mid2 [92-95]. *MID2* and *HCS77* genes have the major *in vivo* role, since deletion of both genes is synthetically lethal, and deletion of each of them results in *pkc1*Δ-like phenotypes, such as sorbitol-remediable cell lysis at

high temperature and high sensitivity to cell wall affecting drugs, while deletion of their homologues causes only minor phenotypes. Mid2 is believed to transduce a calcium signal of the pheromone response [96], as well as signal from cell-wall-perturbations [95;97], whereas the Hcs77 protein probably sends to the Pkc1 kinase signals resulting from membrane perturbations in response to hypo-osmotic and temperature shock [93]. These sensors relay the signal to Rho1, an essential and highly conserved small GTP-binding protein, which directly activates Pkc1 [98;99]. Meanwhile, Rho1 also controls cell wall synthesis by activating the β 1,3-glucan synthase [100;101], and by taking part in the actin cytoskeleton organization during polarized growth [102;103]. The activation of the Pkc1-MAP kinase pathway by environmental stress, α-factor treatment, heat shock or cell wall perturbing molecules leads to the dual phosphorylation of the downstream Slt2 MAP kinase on Thr^{202} and Tyr^{204} residues. It is supposed that this dual phosphorylation results in an active Slt2 kinase [91]. The kinase in turn activates transcription factors, among which Rlm1, a member of the MADS box (MCM1, Agamous, Deficiens, and serum response factor) family of transcription factors [104], which plays a key role in the activation of cell wall related genes [105]. Members of this family are known to dimerize and to be MAPK targets in yeast and mammalian cells [86].

Recent works suggest the existence of three potential pathways that acts in parallel to Pkc1 in the control of cell wall integrity pathway. The first one was identified from a genetic screen aiming to isolate suppressors of *pkc1*Δ and *mpk1*Δ cell lysis. This screen led to the cloning of *BCK2* (*b*ypass of *C k*inase *2*), encoding a serine-threonine rich protein of unknown function and *PPZ1* and *PPZ2*, a pair of functionally redundant protein phosphatases [106]. These genes are likely members of a *SIT4*-dependent pathway that also interacts with cell cycle progression ([107], see below). Two other pathways parallel to Pkc1 were recently identified by genetic interactions observed between *pkc1*, *mpt5* and *ssd1* mutations [108]. *MPT5* encodes a protein involved in cell longevity that is required for growth at high temperature, affects telomeric silencing and is implicated in resistance to starvation [109-111]. Mutants of *MPT5* also harbour phenotypes associated with a weakened cell wall. On the other hand, Ssd1 has homology to several ribonucleases and seems to bind poly(A) mRNA. *SSD1* has been shown to interact genetically with genes downstream of *PKC1* [112]. Sensitivity to cell wall perturbing drugs and effects on cell wall weakening are synergistically amplified by the *mpt5ssd1* double mutation. In addition, synthetic lethality is obtained by mutation of *MPT5*, *SSD1* with *SWI4* or *SWI6*, two downstream targets of the *PKC1-SLT2* pathway [108], and see below). Taken together, these genetic data illustrate the complexity of the cell wall integrity mechanisms and the interconnections of these mechanisms with the cell cycle progression.

Coordination between cell wall biogenesis and cell proliferation

Cell proliferation and cell wall biogenesis must be coordinated to allow budding. The process of bud emergence and bud expansion involves remodelling of the mother cell wall as well as synthesis and assembly of new cell wall components. Coherent with this picture, a periodic transcription has been reported for many cell wall genes with a maximum expression in late G1/early S phase of the cell cycle, coincident with the early stages of budding [113]. Cytokinesis, in turn, requires the expression of genes encoding specific enzymes related to synthesis and degradation of cell wall components [114]. For these two cell cycle steps, the transcription factors involved are Swi4, the

DNA binding subunit of SBF, and Rlm1, a MADS box transcription factor, which are both under the control of the Slt2 MAP kinase [87;105;113-115].

The interrelationship between the Pkc1-MAP kinase pathway and the cell cycle progression, dependent upon the cyclin-Cdc28 (the yeast counterpart of mammalian Cdc2), has been illustrated by several experimental data. It was first shown that a *slt2* mutation enhances the defect in G1-S transition associated with *cdc28* alleles [116], and later on, that Slt2 is specifically activated during periods in which cells undergo polarized growth (i.e. just after the START in G1 phase of the cell cycle), in a manner partially dependent of Cdc28 [117]. The proposed mechanism for this effect was that the activation of Cdc28 at START induces a burst in the production of diacylglycerol, which in turn would activate the yeast Pkc1, although, in contrast to its mammalian counterpart, a direct activating effect of diacylglycerol has not been reported for the yeast enzyme [118]. Pkc1 activation at START promotes three different cellular processes: the transcriptional activation of cell wall synthesis genes [113], actin polarization [103;119], and spindle pole body duplication [120].

Another aspect of the cross talk between Pkc1 and Cln-Cdc28 pathways concerns the control of the transcription factor SBF (for SCB binding factor) by these two kinases. SBF is a heterodimeric complex composed of Swi6, a regulatory subunit, and Swi4 that binds the DNA sequence CACGAAA (SCB, for *S*wi4 cell *c*ycle *b*ox) [121-124]. Swi6 is also part of another cell cycle transcription factor, MBF (for *Mlu*I binding factor), together with the DNA binding protein Mbp1 [125]. Both SBF and MBF activate gene expression at the G1/S transition of the cell cycle, but SBF target genes are predominantly involved in budding, in membrane and cell wall biosynthesis, whereas DNA replication and repair are the dominant functions of MBF activated genes [126]. Control of the SBF factor by the cyclin-Cdc28 complex occurs by a Cdc28-dependent phosphorylation of a serine residue on Swi6, which allows nuclear localization of the protein at the end of the M phase and during G1 phase [127]. Swi4 remains nuclear throughout the cell cycle, but is unable to bind SCB-containing DNA in the absence of Swi6, due to an intramolecular mechanism of auto-inhibition that involves both its DNA binding domain and its Swi6 binding domain [128]. Control of the SBF factor also occurs by another cyclin/Cdc28 kinase complex, which in G2 phase inhibits and possibly inactivates SBF [129;130]. Meanwhile, Pkc1-MAP kinase pathway can control SBF activity at the G1-phase through Slt2-dependent phosphorylation of Swi4 and Swi6 [87], enabling recruitment of SBF to the promoters of some SBF target genes [115]. In addition, it was recently shown that Swi4, independently of Swi6, could activate a subset of genes in a Slt2-dependent manner [115]. Slt2 might relieve the intra-molecular interactions that prevent Swi4 from binding to its DNA binding motif in the absence of Swi6, and thus allow Swi4 to regulate transcription of genes partly independently of the G1 phase. SBF is a key element in the control of cell wall synthesis genes and, while it is a target of Slt2, SBF also acts independently of Pkc1 as indicated by the synthetic lethality between *swi4* and *pkc1* mutants [113].

Knr4 as an element for the coordination between cell wall integrity and cell proliferation

Data given above could lead to the idea that Pkc1 and the cyclin-Cdc28 pathways converge at the level of Slt2 protein kinase. This is by far too simplistic because a deletion of *SLT2* does not harbour phenotypes that are characteristic of a *pkc1* or a *cdc28* mutant. More specifically, the cell lysis defect associated with lack of

Pkc1 is much severe than those caused by any mutation in the downstream MAP kinase cascade, including *SLT2*. These findings argue for the existence of another branch of the Pkc1 pathway that bifurcates just downstream of Pkc1 [88]. To identify genes that function in this hypothetical second branch, a screen for dosage dependent suppressors of *pkc1*Δ and *mpk1*Δ defects has been conducted. The *BCK2* (*b*ypass of *C k*inase *2*) gene, encoding a serine-threonine rich protein was isolated, together with *PPZ1* and *PPZ2*, a pair of functionally redundant protein phosphatases [106]. *BCK2* was also identified as a suppressor of *cln* deficiency (lack of the G1 cyclins Cln1, Cln2 and Cln3) [131;132]. Moreover, it was shown that *BCK2* deletion is synthetic lethal with *cln3* mutation [131]. This effect was attributed to the transcriptional activation of *CLN1* and *CLN2* genes by Bck2 in the absence of Cln3 [132]. Bck2 is thus part of a parallel *PKC1*-pathway that activates, together with Cln3/Cdc28, *CLN1* and *CLN2* during late G1, and this activation was found to be partly but not completely dependent on SBF and MBF [133].

The synthetic lethality between mutations in *PKC1-SLT2* linear pathway and *SWI4* or *SWI6* [92;113], between *BCK2* and *CLN3* deletions [131-133], and the fact that a *bck2*Δ *pkc1*Δ double mutant displays an extremely severe growth defect [106], suggests that *PKC1* and *BCK2* pathways converge to hit common targets or that both pathways are connected at some points to affect common targets. Several arguments in favour of this second hypothesis were obtained and identified Knr4 protein as one of the elements that allows Pkc1 to coordinate cell wall integrity with cell cycle progression. The *KNR4* gene was originally isolated from a recessive mutation giving resistance to the killer toxin from *Hansenula Mrakii* [134], and later on as a multicopy suppressor of many *cwh* (*c*alcofluor *w*hite *h*ypersensitive) mutants [135]. Although there is no proof yet that Bck2 interacts physically with Knr4, all genetic data converge to the idea that these two proteins are part of a complex that is involved in the control of G1-target genes together with Cln3-Ccd28 (see Figure 4). A major evidence for this model is that deletion of *KNR4* is, like that of *BCK2*, synthetically lethal with *cln3* mutation [136]. In addition, the phenotypes associated with deletion or overexpression of these two genes are similar, and they both exhibit synthetic lethal interactions with members of the Pkc1 pathway. However, a genome wide analysis of cells overexpresssing *BCK2* or *KNR4* indicates that Bck2 when it is overproduced, can act independently of Knr4. These independent effects can account for the fact that overexpression of this gene, but not of *KNR4*, can rescue cell lysis of a *pkc1*Δ [136].

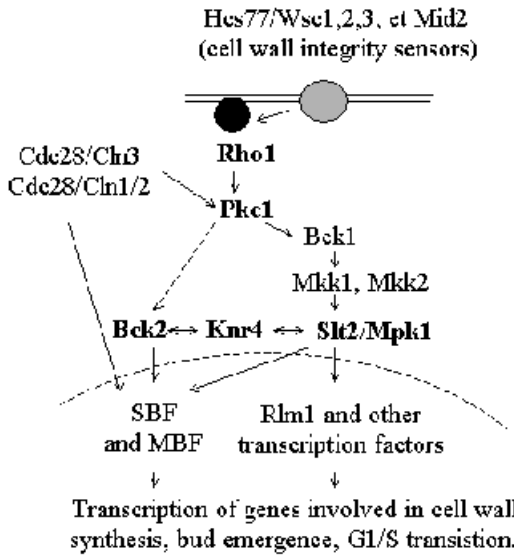

Figure 4: A model proposed for the function of Knr4 protein in the Slt2-MAP kinase pathway of *Saccharomyces cerevisiae*. The dual interaction of Knr4 with Bck2 and Slt2 is one of the elements by which Pkc1-pathway coordinates cell wall integrity with cell growth. Solid arrows indicate activation, double arrowhead indicates protein interaction, and dashed arrow is still suggestive interaction.

Knr4 physically interacts with the MAP kinase Slt2, and is required for the strong increase of Slt2 kinase activity induced by

heat shock [137]. Remarkably, deletion of *KNR4* does not impair signalling through the *PKC1* pathway that leads to dual phosphorylation of Slt2 protein, but it prevents this kinase to efficiently phosphorylate some of its substrates. Although all the targets of Slt2 are not known yet, the transcription factors Rlm1 and SBF have been reported to be among them. In cells defective for *KNR4*, the transcriptional activity of Rlm1 is dramatically reduced whereas the transcriptional activity of SBF is strongly increased, and the phosphorylated (and probably) activated form of Swi6 is more abundant. Thus, during vegetative growth, Knr4 seems to monitor the fine tuning of output signals of the Pkc1-MAP kinase, acting as a switch to favour Rlm1, and hence the cell wall synthesis genes, versus SBF and cell cycle progression (see Figure 4).

Knr4 is a small protein conserved among yeasts, which localizes to sites of polarized growth [135]. Its interaction with Slt2 and Bck2 is only one of the regulatory aspects driven by this protein because other protein interactions with Knr4 have been reported. Knr4 strongly interacts with the tyrosyl-tRNA synthetase encoded by *TYS1*, and this physical interaction seems to be required for dityrosine formation during the sporulation process. This hypothesis was reinforced by the finding that the efficiency of spore formation was drastically reduced in diploid cells homozygous for the disruption of *KNR4* or for a temperature-sensitive mutation of *TYS1* [138]. Other interactions such as with Cin5, a transcription factor of the Yap family, and Bas1, a transcription factor implicated in basal expression of adenine metabolic genes [138;139] have been reported, but not fully analysed yet.

BIOCHEMISTRY AND MOLECULAR ANALYSIS OF THE CELL WALL REPAIR MECHANISM

Yeast cells are living in environmental conditions that can weaken their wall. It is therefore not surprising that they have developed some mechanisms for cell wall rearrangement to combat cell lysis. Consistent with this idea, cell wall damages induced by wall perturbing drugs such as Calcofluor White, caffeine, SDS, zymolyase, or by mutations in cell wall related genes are accompanied by dramatic changes in the molecular architecture of the wall [3;5;83]. Three major responses characterize the so-called ‘cell-wall salvage’ pathway. First, the balance among the cell wall polysaccharides components is modified, with chitin content being the most affected since it can reach up to 20 % of the cell wall mass. Secondly, the type of association among components is changed. For instance, lowering the amount of β-1,6-glucan leads to a larger fraction of the cell wall proteins to become linked directly to β1,3-glucan and chitin, consistent with an increased levels of PIR-proteins. A third response that ensures strengthening of the cell wall is a transient redistribution of the cell wall synthesis and repair machinery, that is normally focused to active growth regions, all over the cell periphery [5].

A consequence of cell wall damages is a considerable increase in chitin [77;140;141] which likely contributes to strengthening the cell wall. It was initially considered that the rise of chitin in response to cell wall defects was consecutive to an activation of the chitin synthase 3 encoded by *CHS3* [142;143]. However, the conclusion of these works was hampered by inconsistencies of data on Chs3. Indeed, abolition of chitin synthesis activation by deletion of *CHS3* in cell wall defective mutants is not a proof that Chs3 is the controlling enzyme in this

mechanism. Reconsidering this question, we actually showed that *GFA1* encoding glutamine - fructose-6-phosphate amidotransferase (Gfa1), the first committed enzyme of chitin biosynthesis pathway, plays a major role in this process. Using the terminology of the Metabolic Control Analysis [144], we showed that in quantitative terms, the reaction catalysed by Gfa1 has a flux-coefficient control in the range of 0.90, indicating that the major control of the chitin metabolic pathway takes place at the level of this reaction. Moreover, our data established that the control of the chitin metabolic pathway is mainly hierarchical [145], *i.e.* dominated by a transcriptional control of *GFA1* [146].

The DNA microarrays and proteomic are powerful technologies that have been recently used to decipher the molecular mechanisms underlying the cell wall repair mechanism. A first attempt was made by Jung and Levin [105] who created a permanent and strong activation of the linear Pkc1-Slt2 MAP kinase cascade using a gain-of-function allele of *MKK1* (*MKK*^{S368P}) that was placed under the galactose-inducible promoter. This work led to the identification of a collection of about 25 genes whose up-regulation was totally dependent upon Rlm1. This approach also brings about some intriguing remarks, since, for instance, many genes previously recognized as being activated by Rlm1 were not found, and *FKS2* was strongly activated in a possible Rlm1-independent manner, although the promoter of this gene harbours a specific Rlm1-DNA binding consensus [147]. Another genome wide study was carried out by comparing expression profiles of a *fks1*Δ mutant with its isogenic wild type [11]. This work also led to the identification of genes whose transcriptional changes were clearly dependent upon the Pkc1 Map kinase pathway. Interestingly, *SLT2* transcript was upregulated, indicating a positive feedback loop of the phosphorylated Slt2 on its own expression. These two genome wide analysis illustrate the role of the Pkc1-MAP kinase cascade in the cell wall repair mechanism, and show that the transcriptional response implicates Rlm1 as well as other transcriptional factors. Therefore, to enlarge this analysis, we conducted a genome-wide survey of genes expression changes caused by five independent cell-wall mutations, namely mutations in genes implicated in cell wall structure (*mnn9, fks1* and *kre6)*, in the interconnection of cell wall components *(gas1)* and in the regulation of cell wall biosynthesis *(knr4)*. Overall, roughly 300 genes were responsive with transcriptional changes ranging from 1.4 to more than 10-fold. The repartition of the differentially expressed genes into functional categories revealed an enrichment of genes that belong to energy metabolism and cell defense. A two-dimensional hierarchical clustering method identified a major group of about 100 genes that were up regulated in the five cell wall mutants, from which roughly 30 % have no annotated function, and less than 10 % were known to be controlled by the *PKC1*-dependent cell integrity pathway. Using available softwares, over-represented DNA sequences were identified in the upstream non-coding regions of these genes. They correspond to binding sites of known transcriptional factors involved in activation of cell wall genes (Rlm1), in stress and heat shock responses (Msn2/4 and Hsf1), in the cell cycle (Sok2), as well as in carbon and phosphate metabolism (Gcr1 and Pho4). Interestingly, a novel putative 6-bp regulatory motif was found in the promoters of 30 % of the co-regulated genes. This motif was shown to be functional by site-directed mutagenesis, but its implication in cell wall assembly as well as the transcription factor it may bind remains to be established. To conclude, these global analyses demonstrate that the cell wall compensatory mechanism likely encompasses the coordinated action of multiple transcription factors responsible

of the expression patterns in response to cell wall defects [148].

BIOTECHNOLOGICAL VALUES OF THE CELL WALL COMPONENTS

Yeast is a eucaryote, which represents an ideal tool because of its short generation time, its well-known metabolism and the possibility to easily carry out genetic modifications. Moreover, yeast is a GRAS ("*G*enerally *R*egarded *A*s *S*afe") organism, so it can be legally used in agro-food and pharmaceutical productions. The cell wall components present several advantages towards these issues that we will revise shortly below.

Biotechnological values in traditional use of yeasts

Yeast flocculation and flotation in beverages

Flocculation refers to an asexual cellular aggregation when yeast cells adhere, reversibly to one another to form microscopic flocs which sediment out of the suspension. Conversely, yeast flotation defines the ability of non-aggregated yeast cells to trap $C0_2$ bubbles in a fermenting liquid and form a film or vellum at the top of fermentation vessels [149;150]. Both phenomena are highly relevant for the production of several yeast-fermented products. Flocculation and flotation are governed by many external (physicochemical, environmental) and intrinsic (genetic) parameters that are still poorly understood. Biochemical analysis of the flocculation process has identified at least two types of molecules on the yeast cell surface that are responsible of the flocculation. One type is the mannan core of the mannoproteins, and the other is flocculins (surface glycoproteins) that are encoded by *FLO* genes [149-152]. The overall structure of flocculins, encoded by *FLO1, FLO5, FLO10* and *FLO11,* comprise an amino-terminal domain containing a hydrophobic signal sequence and a carboxyl-terminal domain with homology to the GPI-anchor proteins, separated by a domain of highly repeated sequences rich in serine and threonine residues. These proteins bind to mannans on the surface of neighbouring cells, leading to the cross binding of cells and ultimately the formation of flocs, each consisting of several cells. Cell surface charge and hydrophobicity have also been implicated with flocculins to facilitate flocculation. Cell flotation is associated with the formation of a vellum. This structure is likely the result of a complex association of highly glycosylated cell-surface proteins as indicated by genetic and biochemical studies [149;150;153;154]. For both phenomena, the relevant genetic and metabolic mechanisms need to be worked out in order to develop strategies for controlling flocs and vellum formation during fermentation.

Yeast polysaccharides in winemaking

Besides many polysaccharides present during winemaking and that arise from grapes or from deceased microorganisms, mannoproteins from the wall of *Saccharomyces cerevisiae* have some beneficial effects on fining and clarification during wine production by ensuring physicochemical stability of the end product, for at least four reasons [155]. Firstly, mannoproteins have a positive effect on the tartrate stability [156], which likely limits crystal formation [157]. Secondly, mannoproteins have a noticeable influence on the protein stability of white and rosé wines [158] and display the ability to increase the stabilisation of aroma components [159]. Moreover, mannoproteins are supposed to bind to tannins, which reduce the astringency of wines [160]. Another reason comes from the finding that mannoproteins affects positively the onset and development of malolactic fermentation [161;162]. In

practice, some winemakers add to the wine broth commercially available β-glucanases that release mannoproteins, to enhance natural effect of endo-β-glucanases obtained after yeast autolysis [163]. Due to the beneficial effect of mannoproteins on wine quality, future efforts should be concentrated on methods that increase the mannoproteins content of the yeast cell wall and favour yeast autolysis at some stage of the wine process [149;150].

Cell wall components as a benefit for human health

Yeasts extracts have been used since a long time for cosmetic and pharmaceutical purposes, and the first pharmaceutical 'Zymosan' was just composed of a raw yeast cell wall preparation that exhibited an immune-stimulating activity [164]. It was then recognized some years later that β-glucans of the yeast *Saccharomyces cerevisiae*, have immune-suppressive activity [165-168] probably due to their effects to activate the macrophages [169;170]. β1,3-glucans display other interesting properties as adjuvant added to anti-infective agents [171;172], to anti-neoplastic agents [173;174], to topical agents [175;176], to vitamins C derivatives, or to cholesterol reducing agents [177], or when administrated to patient under radiotherapy treatment [178]. β1,3-glucans might also be interesting active compounds suitable for cosmetic and dermatological applications [179], as indicated by their presence, as a carboxymethylated derived form, in the composition of cosmetic lotions and sunscreens. Due to these empirical though beneficial effects, β1,3-glucans, a product mainly obtained from yeast wall, has appeared on the market [180]. Recent researches have focused on the potential applications of acid treated yeast cell wall (AYC) as a novel coated material in pharmaceutical products [181;182]. Yeast cell wall mannans are suspected to stimulate the immune system, since they can induce a pyrogenic prostaglandin-dependent response by direct injection in rats [183;184]. Moreover, mannoproteins present an effective bio emulsifier power potentially interesting for a wide range of medical, pharmaceutical applications [185].

Animal Nutrition and Prevention against Mycotoxins

Yeast cells and yeast cell walls are currently added to animal diets in order to facilitate the digestion and to protect them from pathogens. On the other hand, one of the most important applications resides in the ability of cell wall to protect animal from mycotoxins. These latter are secondary products of fungal metabolism that may be produced in contaminated feeds during production and storage. Aflatoxins and fusarium toxins are responsible of liver damages, decrease of reproductive performances, tumor formation, immuno-suppression. A trivial method for attenuating the effect of mycotoxins is based on the use of materials that can absorb mycotoxins in animal feeds, without any secondary effects. This allows the toxin to pass through the animal's digestive tract without being retained. The carbohydrate complexes of the yeast cell wall was reported to express this capacity when administrated to chicken [186;187]. Modifications in the manufacturing techniques have allowed the production of more efficient yeast cell wall preparation with the ability to bind a wide range of mycotoxins, such as the Mycosorb™, a yeast cell wall-derived glucomannan product [188]. A better understanding of biochemistry related to the interaction of mycotoxins with the cell wall components should be important for development of advanced mycotoxins adsorbents in the future [189]. Genetic modifications of the yeast cell wall or strain selection could lead to the discovery of cell wall structures able to bind an even wider range of mycotoxins with even higher affinity.

Cell Wall Engineering

The attachment of homologous or heterologous proteins to the cell surface to create new metabolic abilities of cells was investigated only a few years ago, thanks to the identification of GPI-structure as a mean to anchor proteins to cell wall β-glucans [190]. As an example, a chimeric protein bearing the *Rhizopus oryzae* glucoamylase has been fused to the C-terminal half of the yeast α-agglutinin, a protein involved in mating and which is covalently anchored to the cell wall. The transformed strain expresses the chimeric enzyme at the cell surface, allowing growth on starch as the sole carbon source [191]. A similar strategy was followed with many other proteins like α-amylase from *Bacillus stearothermophilus* [192], cellulase and lipase [193;194]. This strategy was also applied to generate polypeptide libraries and then to screen by cell sorting techniques those proteins showing the best affinity for specific ligands [190;195-197]. The expression of antigens at the cell-surface of yeast is an attractive approach for the development of recombinant live vaccines whose efficiency was already proven in the treatment of diarrhoea [198]. Although first trials of this approach was disappointing regarding the low antigenicity of the immobilized protein, more extensive engineering of the outer surface should lead to the production of a cheap and safe oral vaccine [199;200]. We can also foresee many more applications, such as immobilization of proteins with specific binding properties for purification, bioseparation and detection of a wide range of chemical and biological compounds.

Strategies to weaken the yeast cell wall

Yeast cells surrounded by a 'thin cell wall' would be of great value for those agro-factories that want to extract flavours and macromolecules from the intracellular compartments, since rupture of cell wall by mechanical devices is time and money consuming in comparison to downstream steps of the process. The biotechnological challenge has been to generate by metabolic engineering strains that possesses a wall easy to break, without modifying the physiological performance of the industrial yeast strains [201]. Until now, no compromise solution between reducing cell wall and keeping intact the physiological parameters has been achieved [202]. For instance, mutations in *PKC1* dependent cell wall integrity pathway cause pleiotropic consequences for industrial applications, whereas strains that produce reduced amount of a β-glucans or mannoproteins grow very slowly and induce a cell wall rescue mechanism that actually leads to an increase of cell wall strength [140]. An alternative strategy would be to impair some of the covalent linkages that link together the cell wall components.

Ideal target for antifungal agents ?

Fungal infections in humans range from superficial and cutaneous, like dermatophytose, to deeply invasive, such as candidiasis and cryptococcosis [203]. In the last 25 years, the frequency of systemic, often life-threatening, fungal infections has dramatically increased due to the proliferation of patients who are severely immuno-compromised by cancer chemotherapy, organ transplantation, HIV infection. Current available antifungal drugs for serious infections are either fungistatic (azoles) or fungicidal (polyenes) but also toxic for the host. Cell wall-acting antimicrobial agents are inherently selective and fungicidal, features that make them particularly attractive for clinical development. Three classes of such compounds, targeted respectively to chitin synthase (nikkomycins), β1,3-glucan (echinocandins) and mannoproteins (pradimicins) have entered in clinical development. Nikkomycins act competitively as substrate analogous of the

UDP-*N*-acetyl-glucosamine in preventing chitin synthesis [204], echinocandins are fatty acid derivatives of cyclic hexapeptides that inhibit non competitively β1,3-glucan synthesis, and the pradimicin family of antifungals exerts its selectivity by calcium-dependent binding of cell surface mannoproteins, which leads to cell wall leakage and loss of viability [205]. While nikkomycins and pradimicins are no longer in development due to their low '*in vivo'* efficiency, the echinocandins have emerged as clinically useful and three compounds, caspofungin, micafungin and anidulafungin are in late development (clinical phase II and III) [206]. Although these products are quite promising, continued efforts are necessary to find even better targets at the cell surface. The enzymes involved in cross-linking of the cell wall modules are likely good candidates, but this requires much more attention at the biochemical and enzymological levels, in order to develop high throughput assays to screen natural or chemical libraries that are already available in many pharmaceutical companies.

CONCLUSIONS AND PERSPECTIVES

A clear picture of the cell wall architecture is now emerging thanks to recent biochemical and genetic studies carried out by many public and private research groups worldwide. This knowledge is very important as it will orient our future research programs aimed at optimising cell wall components production for nutrition and to find better antifungal targets. It is clear that the urgent step today is to identify the cross-linking enzymes, the precise sequence by which these cross-linking reactions occur, and their genetic and metabolic regulation. The molecular deciphering of the cell wall repair mechanism is only emerging, and a key issue will be to clarify the cross talks that certainly exist between the different signalling pathways, namely PKC1-MAP kinase, HOG-MAPK, RAS-cAMP, and probably others like TOR [82;207]. Although *S. cerevisiae* is not known to be pathogen, most of the information collected on this organism will surely facilitate our understanding of the physiology of pathogenic fungi, like *Candida albicans*.

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