Review

‘Strengthening the fungal cell wall through chitin–glucan cross-links: effects on morphogenesis and cell integrity’

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Summary

The cross-linking of polysaccharides to assemble new cell wall in fungi requires transglycosylation mechanisms by which preexisting glycosidic linkages are broken and new linkages are created between the polysaccharides. The molecular mechanisms for these processes, which are essential for fungal cell biology, are only now beginning to be elucidated. Recent development of in vivo and in vitro biochemical approaches has allowed characterization of important aspects about the formation of chitin–glucan covalent cell wall cross-links by cell wall transglycosylases of the CRH family and their biological function. Covalent linkages between chitin and glucan mediated by Crh proteins control morphogenesis and also play important roles in the remodeling of the fungal cell wall as part of the compensatory responses necessary to counterbalance cell wall stress. These enzymes are encoded by multigene families of redundant proteins very well conserved in fungal genomes but absent in mammalian cells. Understanding the molecular basis of fungal adaptation to cell wall stress through these and other cell wall remodeling enzymatic activities offers an opportunity to explore novel antifungal treatments and to identify potential fungal virulence factors.

Introduction

Fungal cells are surrounded by the cell wall, an essential structure that determines cell shape and protects cells against bursting caused by internal turgor pressure or adverse effects of the environment (Cid et al., 1995; Levin, 2011). In addition to the protective effect of this structure and its role in cellular morphogenesis, fungal cell wall polysaccharides specifically interact with the immune system receptors, thus playing a crucial role in host defense responses (Latgé and Beauvais, 2014).

The essentiality of the cell wall for fungal viability together with the fact that their components are not present in mammals makes this structure as one of the most attractive targets for therapeutic intervention against fungal infections (Tada et al., 2013). Although appearing relatively static, the fungal cell wall is however very dynamic and continuously changes in polysaccharide composition and distribution to adapt its growth to environmental conditions. Therefore, characterization of the molecular mechanisms of cell wall assembly and remodeling is essential, not only for a better understanding of fungal cell biology, but also for the identification of specific fungal–host interactions and the design of novel antifungal strategies.

Cell wall biogenesis: synthesis and cross-linking of cell wall polymers

In spite of several differences in cell wall composition between fungal species, the overall structure of the fungal cell wall is conserved. The backbone of this matrix is composed of a branched β-1,3-glucan cross-linked to chitin (Lesage and Bussey, 2006; Orlean, 2012). This glucan–chitin complex is covalently bound to other polysaccharides, the composition of which varies between different fungal species (Free, 2013). All components are covalently linked to each other to form a hardy network that is responsible for the mechanical strength of the cell wall structure. In Saccharomyces cerevisiae, the non-reducing end of the β-1,3-glucan chains is attached to the reducing end of the β-1,6-glucan by an uncharacterized link and to the reducing end of the chitin by a β-1,4-glycosidic linkage (Kapteyn et al., 1996; Kollar et al., 1995; Kollar et al., 1997). β-1,6-glucan is also attached to the chitin through β-1,3-linked oligoglucose-residues that branch off the glucan. Cell wall mannoproteins,

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which include proteins involved in adhesion, enzymes involved in cell wall remodeling, structural proteins and somatic antigens (Cabib et al., 2007; Dranginis et al., 2007; Mouyna et al., 2000) are linked to β-1,6-glucan through a glycosylphosphatidylinositol (GPI) anchor (Ferguson et al., 2009). β-1,6-glucan is present in both S. cerevisiae and Candida albicans (Lesage and Bussey, 2006; Ruiz-Herrera et al., 2006) whereas it is absent in Aspergillus fumigatus (Aimananda et al., 2009). In contrast, galactomannan and β-1,3-1,4-glucan are present in Aspergillus but not in yeast (Latgé, 2007). This diversity has been selected over evolutionary time and is likely to contribute to the ability of the different fungi to survive at different biological niches (Free, 2013).

Chitin and β-glucans are synthesized by plasma membrane associated synthases, which extrude newly formed linear polymer molecules through the membrane into the extracytoplasmic space (Aimananda et al., 2009; Cabib et al., 1983; Durán et al., 1975; Montijn et al., 1999; Shematek and Cabib, 1980). Mannoproteins are synthesized in the ER and then transported to the cell surface through the secretory pathway where a GPI anchor, N-linked and O-linked oligosaccharides are added (Lesage and Bussey, 2006; Orlean, 2012).

The final stage of cell wall construction and remodeling is the creation of covalent cross-links between individual cell wall components to form a three-dimensional cell wall fabric. Effective cell wall remodeling during cell growth requires elongation, branching and cross-linking of newly synthesized polysaccharides to the preexisting cell wall core. Linear cell wall components are synthesized at the plasma membrane and extruded outside into the cell wall space. Therefore, these processes take place in situ at the periplasmic space and in the cell wall whereby the energy needed for creation of new glycosidic bonds must be supplied from the breakage of preexisting bonds; hence, the cross-linking must be catalyzed by transglycosylases (Cabib et al., 1988).

To date, although a plethora of fungal glycoside hydrolase activities has been described (CAZY at http://www.cazy.org/) only few fungal proteins acting as transglycosylases and presumably involved in cell wall remodeling have been characterized. Elongation of the β-1,3-glucan is performed through the action of the Gas1/Ge11/Phr1 family of β-1,3-glucanases, in vitro acting as β-1,3-glucanosyl transferases (Mazán et al., 2013; Mouyna et al., 2000). These enzymes, whose structure has been elucidated (Hurtado-Guerrero et al., 2009), split β-1,3-glucan and transfer the newly generated reducing end to the non-reducing end of another β-1,3-glucan molecule. In addition, a branching activity has been ascribed in vitro for Bgt1 and Bgt2 in A. fumigatus, although a true role for this activity in vivo is lacking (Gastebois et al., 2010). These proteins, which are orthologs of the S. cerevisiae Bgl2, remove laminariobiose units from non-reducing ends of β-1,3-glucan and transfer them to non-reducing ends of other β-1,3-glucan molecules with formation of a β-1,6-glycosidic linkage. In vitro, exo-β-1,3-glucanases from C. albicans belonging to GH5 family, also described in S. cerevisiae, Schizosaccharomyces pombe (Duerfas-Santero et al., 2010) and A. fumigatus (Mouyna et al., 2013), not only hydrolyze β-1,3-glucan but also catalyzes an efficient transglycosylation reaction (Mackenzie et al., 1997; Stubbs et al., 1999).

Regarding the process of cross-linking between cell wall polysaccharides, the enzymes catalyzing the formation of cross-links between chitin and glucan belong to the conserved family CRH of fungal cell wall transglycosylases (Cabib, 2009; Cabib et al., 2007; Rodríguez-Peña et al., 2000). In this short review we are summarizing the knowledge gained in the last few years about their cross-linking activity both in vivo and in vitro, their function in morphogenesis and maintenance of cell integrity, and pointing out to their potential use as antifungal targets.

**CRH proteins create links between chitin and β-glucan both in vivo and in vitro**

Chitin, a linear polysaccharide composed of β-1,4-linked N-acetylglucosamine residues, makes up between 1 and 15% of the fungal cell wall mass, with yeast species having 1–2% chitin and filamentous fungi having up to 15% (Free, 2013). In spite of being a minor component of the yeast cell wall, chitin is essential for cell survival because of its significant contribution to the rigidity and strength of the cell wall and particularly because of its role in cell division. This polysaccharide is laid down at three locations: a ring at mother-bud neck, the primary septum and throughout the lateral cell wall. Characterization of the cross-linking between chitin and glucans at these locations was possible because of the development of novel biochemical approaches to cell wall analysis (Cabib, 2009; Cabib and Durán, 2005). Chitin can be labeled specifically by growing cells in the presence of $^{14}$C-glucosamine. By treating or not treating portions of the labeled cell walls with different hydrolytic enzymes, such as β-1,3-glucanase, β-1,6-glucanase and chitinase and then comparing the respective factions by size exclusion chromatography after solubilization by carboxymethylation, it is possible to quantify the different chitin-associated factions. Using this approach together with two additional methods it was found that in a yeast wild type strain approximately 40% of the chitin is free (not covalently bound to glucans) whereas β-1,3-glucan and β-1,6-glucan chitin associated factions represent 40–45% and 15–20% of the total chitin, respectively (Cabib, 2009; Cabib and Durán, 2005; Cabib et al., 2007) (Fig. 1A). Moreover, the association of chitin with different polysaccharides varies depending on location: at the neck most of the chitin is linked to β-1,3-glucan whereas in the lateral cell wall the attachment to β-1,6-glucan predominates (Cabib and Durán, 2005).
The first suggestions about the enzymes responsible for the formation of chitin–glucan linkages came from studies on proteins belonging to the CRH family (for Congo Red hypersensitivity). The three members of this family in *S. cerevisiae*, Crh1, Crh2 and Crr1, exhibit significant homology with bacterial β-1,3/1,4-glucanases (Planas, 2000) and xyloglucan endotransglycosylases/hydrolases (XETs/XTHs) from plants (Rose *et al*., 2002) and have been included in the glycohydrolase family 16 (GH16) at the CAZy database (Cantarel *et al*., 2009). Crr1 is involved in spore cell wall biogenesis (Gómez-Esquer *et al*., 2004), whereas Crh1 and Crh2 function during vegetative growth (Rodríguez-Peña *et al*., 2000). Glucan insolubility in alkali is because of its binding to chitin (Kollar *et al*., 1995) and a double mutant *crh1Δ crh2Δ* strain showed a clear increase in the alkali-soluble glucan fraction. This, together with a similar localization of these proteins to that of the chitin throughout the cell cycle, pointed to these proteins as possible chitin–glucan cross-linkers (Rodríguez-Peña *et al*., 2000).

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**Fig. 1.** Formation of chitin–glucan cross-links at the fungal cell wall.  
A. Distribution of chitin fractions in the wild type and different *crh* mutants (Cabib, 2009).  
B. The transglycosylase reaction catalyzed by Crh1 and Crh2 proteins involves the cleavage of the β-1,4 linkages of the nascent chitin (upper panel) or insoluble chitin already synthesized and deposited at the cell wall network (lower panel) and subsequent attachment of the polysaccharide fragment to the glucan acceptor, either β-1,3-glucan or β-1,6-glucan. Upper panel: nascent chitin is cut by Crhs (a). Then, the newly created reducing end, still bound to plasma membrane, is cross-linked to the glucan acceptor (b). Finally, an endochitinase (EChp) or Crh activity should be necessary to detach the hybrid product from the plasma membrane (b and c). Steps depicted in a–c are associated to the same chitin synthase complex. Lower panel: Crh enzymes would also cut chitin already synthesized and detached from the plasma membrane, transferring chitin fragments directly to the glucan acceptor. For β-1,3-glucan, the reducing end of the chito-oligosaccharide is bound to the non-reducing end of the glucan. For β-1,6-glucan, the reducing end of the chitin is bound to a side-branch of β-1,3 glucose of the glucan. Cyt: Cytoplasm; PM: Plasma membrane; PE: Periplasmic space; CW: Cell wall.  
C. General scheme of a transglycosylase reaction which involves a breakage of the glycosidic bond in the polysaccharide molecule acting as donor and linking the newly formed fragment by their newly created reducing end to hydroxyl groups at the non-reducing end of other polysaccharide molecule acting as acceptor with the formation of a new glycosidic bond (Cabib *et al*., 1988). The new glycosidic bond must be formed at the expense of the energy released by breaking preexisting linkages. For *in vitro* transglycosylation assays, the acceptor can be labeled and the amount of label transferred from the acceptor into the hybrid product by the transglycosylating enzyme determined. Circles correspond to glycosyl units, reducing-end units are shown as triangles and stars represent the label, usually a radioactive or fluorescent tag.
Later on, analysis of chitin–glucan cross-links by characterization of free chitin and β-1,3-glucan and β-1,6-glucan chitin associated fractions in isolated cell walls of wild-type and crh mutants demonstrated that Crh1 and Crh2 are responsible for the covalent cross-links between chitin and either β-1,6 or β-1,3-glucan in vivo (Cabib, 2009; Cabib et al., 2007). As shown in Fig. 1A, both glucan-associated fractions are partially reduced in a crh1Δ strain, more reduced in a crh2Δ strain and completely absent in a double mutant crh1Δ crh2Δ where all the chitin is free (Cabib, 2009; Cabib et al., 2007). Although the activity of Crh2 seems to be more relevant in vivo, both proteins Crh1 and Crh2 show a redundant activity. Therefore, in the absence of any of them, the other one is able to transglycosylate the corresponding polysaccharides. The chitin in the double crh1Δ crh2Δ mutant has a higher molecular weight than any of the chitins from the wild-type, in agreement with the fact that this chitin is not split and its fragments not transferred to an acceptor in this case. Moreover, the chitins cross-linked to β-1,6 and β-1,3-glucan are smaller than the free chitin (Cabib et al., 2007), favoring the idea that relatively short fragments of the nascent chitin are directly transferred to the acceptor by the Crh proteins (Fig. 1B).

Further insights into this transglycosylation system came from the development of in vivo and in vitro assays. In the in vivo assay, sulforhodamine (SR)-labeled oligosaccharides derived from β-1,3- and β-1,6-glucans were incorporated into the cell walls at bud scars and at lower levels in the lateral cell walls of growing cells, thus functioning as artificial acceptors for the cross-linking reaction that takes place in the periplasmic space (Cabib et al., 2008; Mazán et al., 2013). A minimal size of three glucoses was required for the acceptor oligosaccharide and the cross-linking efficiency increased with the length of the oligosaccharide chain from 3 to 7 (Cabib et al., 2008). The cross-linking of both glucans in this assay was dependent on both enzymes, Crh1 and Crh2, although to a higher extent on the latter, in agreement with a more relevant role of Crh2 in the cross-linking (Blanco et al., 2015; Mazán et al., 2013). Moreover, the oligosaccharide incorporation was blocked in a crh1Δ crh2Δ strain.

Simultaneous incubation of yeast digitonin-permeabilized cells with UDP-GlcNAc and SR-labeled laminarooligosaccharides led to a massive incorporation of fluorescence into the cells, which is dependent on the Crh proteins. Removal of UDP-GlcNAc caused a disappearance of the fluorescence indicating that chitin formation is necessary for oligosaccharide binding (Cabib et al., 2008). Moreover, the transglycosylation reaction was also operative in isolated cell walls, suggesting that formation of chitin–oligosaccharide complexes in vivo can be achieved by utilization not only of nascent chitin but also of preformed chitin (Fig. 1B). In the first case, nascent chitin would be cut by Crhs, and the newly created reducing, still bound to plasma membrane (PM), would be cross-linked to the glucan acceptor, either β-1,3 or β-1,6-glucan (Fig. 1B, upper panel). An endochitinase activity should be necessary in this case to detach the hybrid product from the PM. Alternatively, the growing hybrid chain could be detached from the PM by repeated attack by Crh proteins. In the latter, the Crh enzymes would use chitin already synthesized and detached from the plasma membrane, transferring chitin fragments including the non-reducing end directly to the glucan acceptor (Fig. 1B, lower panel).

For an in vitro assay to measure transglycosylase activity of Crh recombinant proteins, a system was devised where the natural donor, nascent chitin, was replaced by the soluble carboxymethyl-chitin (CM-chitin). The transglycosylase reaction involves a breakage of the glycosidic bond followed by its reformation at another place. Because there is no net change in the number of reducing groups in the system, the conventional reductometric methods for the assessment of transglycosylase activity cannot be employed. Instead, it is possible to label the acceptor and then determine the amount of label transferred from the acceptor into the hybrid product by the transglycosylating enzyme (Fig. 1C). For a recent overview of transglycosylation assays see Franková and Fry (2015). As acceptors for Crh1 and Crh2 served the fluorescent SR-labeled oligosaccharides derived from β-1,3-glucan, β-1,6-glucan and from chitin, although with different efficiencies, in agreement with the in vivo transglycosylation reaction.

The products of transglycosylation were hybrid polymer molecules composed of the respective SR-labeled oligosaccharide acceptor and a portion of the donor polysaccharide attached to its non-reducing end (Fig. 1C) (Mazán et al., 2013). Soluble N-acetyl chitooligosaccharides of DP ≥ 5 served as the oligosacryol donors as well, whereby the minimal number of intact hexopyranose units required by Crh1 and Crh2 in the molecule of the acceptor oligosaccharide was two. Kinetic parameters, K_m, k_{cat} and k_{cat}/K_m obtained with SR-laminarin tetraose and SR-N-tetraacetyl chitotetraose acceptors, showed that chito-derivative binds to the Crh enzymes more readily than its SR-β-1,3-linked analogue derived from laminarin. These findings suggest that Crh1 and Crh2, in addition to their function as chitin–glucan transglycosylases, could also act as homo-transglycosylases in vivo mutually joining and elongating nascent chitin chains, but this is merely speculative (Mazán et al., 2013). At present, we do not have an explanation for the lack of specificity of Crhs in vitro towards acceptors. It seems that, in general, the enzymes accept all substrates composed of beta-linked glucopyranosyl units. With alpha-linked glucopyranoses, such as starch or maltooligosaccharides, the enzymes are inactive. The loose specificity of the acceptor site is difficult to explain unless we
get more information about the tertiary structure of these enzymes.

**Crh transglycosylases are very well conserved in fungi**

The Crh enzymes are unique to fungi and the genes encoding them are very well conserved in fungal genomes. These enzymes, like other glycosyl hydrolases, are encoded in multigene families which provide a redundancy of cross-linking activity to allow a fully functional cell wall remodeling. As already mentioned, in *Saccharomyces* there are three members of the CRH family, namely Crh1 and Crh2 working in vegetative growth, and Crr1 which appears to have a function in spore cell wall biogenesis. Three members are also encoded in the genome of *C. albicans*, Utr2, Crh11 and Crh12 (Fig. 2A). Based on the phenotypes of mutants deleted in these genes together with localization studies and alterations in the composition of their cell walls, it has been suggested that *Candida* orthologs would develop similar functions to those of the *Saccharomyces* by connecting glucan and chitin (Pardini *et al.*, 2006). Five orthologs have also been identified in the genomes of *A. fumigatus* and *Neurospora crassa*, respectively (Fig. 2A). Interestingly, chitin is absent in the walls of the fission yeast *S. pombe* and this organism lacks homologs in the CRH family.

All these proteins have been included in the glycoside hydrolase family 16 (GH16) of the CAZy database together with other fungal, bacterial and plant enzymes with unrelated functions that share sequence and structure similarities (Cantarel *et al.*, 2009). All of them exhibit a GH16 domain with a highly conserved catalytic region (DE(I/L)DXE) (Fig. 2B) and a secretory signal peptide at the N-terminus. Additionally, the majority of them contain a Ser/Thr-rich region and a GPI attachment signal necessary for the anchoring to the plasma membrane. In addition, a family 18 chitin-binding domain [carbohydrate-binding module 18 (CBM18)] is also present in specific members of the family (Fig. 2A).

Multiple alignments of putative catalytic domains of Crh1, Crh2 and their fungal orthologs in *C. albicans*, *A. fumigatus* and *N. crassa*, show two glutamic residues (E134 and E138 in Crh1), two aspartic acid residues (D133 and D136) respectively.
in Crh1) and one glycine (G141 in Crh1) that are conserved in all of these enzymes (Fig. 2B). Site-directed mutagenesis of amino acid residues at the catalytic domain of S. cerevisiae CRH1 and CRH2 genes and a respective functional analysis revealed that conserved residues within the catalytic domain acting as nucleophile and general acid/base residues (Fig. 2B) are essential for their transglycosylase activity (Blanco et al., 2012; Blanco et al., 2015). Therefore, Crh1 and Crh2 would act as transglycosylases operating via a double displacement mechanism with retention of the anomericity of the formed glycosidic bond (Davies and Henriassat, 1995; Rye and Withers, 2000; Sinnott, 1990). The transglycosylase reaction catalyzed by these proteins involves the cleavage of the β-1,4 linkages of the chitin and subsequent attachment of the fragment of the donor molecule through the newly formed reducing end onto the OH-group of the acceptor molecule, presumably by a β-1,4-glycosidic bond (Blanco et al., 2015) (Fig. 1B). The nature of the newly formed bond is indicated by the fact that it can be hydrolyzed by purified chitinase, and it has been confirmed by MALDI-TOF mass spectrometric analysis of the hybrid products (Mazán et al., 2013). In contrast to other retaining glycosyl hydrolases that retain glycosyl acceptors, Crh1 and Crh2 do not form a covalent intermediate. The transglycosylation reaction catalyzed by Crh1 proceeds through a covalent intermediate, while Crh2 disengages from the donor substrate with the acceptor linked through the newly formed bond (Mazán et al., 2007; Mazán et al., 2013). The transglycosylation reaction in vitro could be developed both on nascent and already synthesized chitin, the exochitinase activity exerted by Crh2 could be involved in the transglycosylation of the latter chitin.

Redundancy is one of the properties of the enzymatic machinery involved in cell wall biogenesis and remodeling. Thus, these enzymes are usually encoded by multigene families which provide the cell wall with the capacity necessary to synthesize and cross-link its structural components under many different biological circumstances (Free, 2013; Orlean, 2012). This probably contributes to the ability of fungi to survive under many different environmental conditions. In S. cerevisiae, in the absence of one enzyme, Crh1 or Crh2, the other one is able to transglycosylate the corresponding polysaccharides (Cabib et al., 2007; Mazán et al., 2013), suggesting a redundancy in the activity of these proteins. Despite their redundant function, analysis of the chitin covalently attached to β-1,3-glucan and β-1,6-glucan and transglycosylation activity in crh1Δ and crh2Δ strains suggests that the activity of Crh2 is predominant in vivo (Cabib et al., 2007; Mazán et al., 2013). This could be explained, at least in part, by differences in gene expression between CRH1 and CRH2 (Rodríguez-Peña et al., 2000).

However, both proteins have different modes of action to process chitin molecules in vitro probably reflecting different biological necessities. As true transglycosylases, Crh enzymes exhibit low hydrolase activity that is accelerated in the presence of oligosaccharide acceptors (Mazán et al., 2013). Chitinolytic activities of both enzymes have been measured in vitro by different assays. Both enzymes are able to degrade chitin and produced 4-methylumbelliferone more readily from 4-MU-(GlcNAc)₃ than from the disaccharide derivative, suggesting that both function as endochitinases. However, whereas no N-acetyl-β-D-glucosaminidase activity was detected for Crh1, a very low but measurable activity was detected for Crh2 with this substrate, suggesting that both enzymes interact differently with the donor substrate: Crh1 attacks the donor molecules at random along the whole length of the donor polysaccharide whereas Crh2, also attacks molecules of the donor substrate chitin preferentially from their non-reducing end (Mazán et al., 2013). Interestingly, because the transglycosylation reaction in vivo could be developed both on nascent and already synthesized chitin, the exochitinase activity exerted by Crh2 could be involved in the transglycosylation of the latter chitin.

Additionally, the presence of the CBM18 domain (chitin binding module) in Crh2, not present in Crh1, seems to be important. There are not conclusive results about the functional role of this CBM in the activity of Crh2. However, site-directed mutagenesis of specific residues within this domain and functional analysis of the transglycosylation reaction in vivo has revealed that mutations at aromatic residues Y43, Y51, F62 and conserved residues P38, S41 and particularly G49 affected the catalytic efficiency (Blanco et al., 2008). The CBM domain is also present in Crh2 orthologs of C. albicans, A. fumigatus and N. crassa (Fig. 2A), further supporting similar conserved mechanisms of action for these proteins in all fungi. Therefore, the theoretical redundancy between these proteins seems to be nuanced through functional differences between Crh homologs that probably make the process of formation of chitin–glucan cross-links much more effective.

**Chitin–glucan cross-links in the cell wall control morphogenesis**

Morphogenesis is one of the major outstanding problems in biological sciences. It concerns the fundamental question of how biological forms and structures are generated. The fungal cell wall determines cell shape. For this reason, the cell wall of the budding yeast S. cerevisiae, including the
septum which is formed at cytokinesis, has been used as model for morphogenesis (Cabib et al., 2001). The control of growth at the mother-bud neck region is absolutely necessary in this case because the diameter of this region remains the same through the cell cycle. Two structures, the chitin ring and the septin ring, control morphogenesis at this region in a redundant way by preventing cell wall growth (Schmidt et al., 2003).

Recently we have described in detail those mechanisms by which cell wall growth is controlled at the bud neck by the chitin ring (Cabib and Arroyo, 2013). In the lateral cell wall, mannoproteins are bound to the β-1,6-glucan which in turn is attached to the non-reducing end of the β-1,3-glucan, allowing cell wall remodeling and growth. However, chitin at the neck is mainly attached to the non-reducing ends of the β-1,3-glucan competing out the β-1,6-glucan attached to the same sites (Cabib and Durán, 2005). This would prevent the linkage of mannoproteins to the β-1,6-glucan and also the remodeling of β-1,3-glucan itself (Blanco et al., 2012; Cabib et al., 2012; Schmidt et al., 2003), thus stopping cell wall growth at the mother-bud neck. Evidences about the effect of the chitin on β-1,3-glucan remodeling came from the comparison between size-distribution of chitin free-β-1,3-glucan and chitin linked-β-1,3-glucan (Cabib et al., 2012). These experiments suggest that β-1,3-glucan attached to chitin at the neck consists of a high molecular weight population that should not be further remodeled. However, understanding the molecular mechanisms involved will require further cell wall remodeling studies. If the formation of the chitin ring is abolished by mutation or inhibition of chitin synthase 3 in a strain partially compromised in septin ring function, the bud neck expands and further growth is abnormal or eventually stopped (Schmidt et al., 2003). The same morphological defects are seen in cells that have a chitin ring but in which chitin to β-1,3-glucan cross-links have been eliminated by deletion of CRH1 and CRH2 (Blanco et al., 2012). In addition, cells treated with nikkomycin, which inhibits chitin synthesis, are morphologically similar to the crh2Δ mutant (Okada et al., 2014). Therefore, specific covalent linkages between chitin and glucan mediated by the Chr proteins clearly control morphogenesis (Cabib and Arroyo, 2013).

Other transglycosylases have also been described to be important for maintenance of the bud neck size. Rolli et al. (2009) reported an increase in mother cell bud neck diameter in a gas1Δ strain. Increased neck width has also been reported in mutants affected in the mannosyltransferase Mnn10 (Schmidt et al., 2005) as well as in cells treated with echinocandin B, tunicamycin or nikkomycin Z, drugs that block cell wall biosynthesis by different mechanisms (Okada et al., 2014), further suggesting a role for the cell wall to preserve the neck structure. However, in these cases, the molecular mechanisms involved have not yet been elucidated.

In addition to the chitin ring, maintenance of the bud neck size also depends on the septin ring. As in other systems (Orlando et al., 2011), septs may act as barriers, preventing the access of cell wall remodeling proteins to the neck area, but no experimental evidence is available on this point. Additionally, correct localization of Chs3, the chitin synthase III catalytic subunit (DeMarini et al., 1997), Crh2 (Rodríguez-Peña et al., 2002) and other proteins required for cytokinesis and septation depends on septs (Oh and Bi, 2011), in agreement with a possible mechanism by which the septin network would restrict the presence of cell wall remodeling enzymes necessary for the control of morphogenesis at the bud neck. The presence of a double control highlights the importance of maintaining the morphology of the mother cell-bud neck.

Because Chr enzymes are conserved through the fungal kingdom, it will be of great interest to study if similar mechanisms might function in hyphal growth of filamentous fungi. In analogy with the role of the chitin-β-1,3-glucan linkages in the budding yeast, they could be also necessary to block growth in lateral cell walls and maintain the shape of the hyphal tube.

Cross-linking between glucan and chitin and cell integrity: Chs as potential antifungal targets

As a consequence of the redundancy, in most cases, mutations affecting single enzymes of cell wall protein families do not lead to dramatic cell growth phenotypes (Lesage and Bussey, 2006). Even simultaneous deletion of CRH1 and CRH2 in S. cerevisiae (Cabib et al., 2007) or CRH orthologs in C. albicans (UTR2, CRH11 and CRH12) (Pardini et al., 2006) or A. fumigatus (Fang et al., 2015) does not result in the lack of cell viability. This effect is exacerbated by compensatory responses triggered by fungal cells when the structure of the cell wall is compromised. In fact, even in the case of A. fumigatus which possesses a unique β-1,3-glucan synthase, lack of this activity, although leading to drastically reduced growth rate, is not essential for survival because of a compensatory increase of chitin and galactosaminogalactan (Dichtl et al., 2015).

These compensatory responses reflect the very dynamic nature of the cell wall structure which varies constantly to adapt to the environment, to cell growth conditions and particularly to external stress. Treatments with cell wall perturbing agents or deletion of genes involved in cell wall biogenesis elicit rescue mechanisms securing maintenance of cellular integrity and fungal survival (Levin, 2011). These responses were first characterized in S. cerevisiae and lead to, among other effects, a remarkable increase in chitin content, an increase in the bulk of cell wall proteins, changes in the cross-linking between cell wall polymers and a transient redistribution of β-1,3-glucan synthase complex throughout the cell (Delley and Hall, 1999; Kapteyn et al., 1997; Popolo et al., 2001). In addition to an increase in the chitin content, heat shock or caspofungin treatment causes an increase in the β-1,6-glucan polyssacharide fraction and
a partial reduction of β-1,3-glucan, both in S. cerevisiae and C. albicans (Schiavone et al., 2014). Part of these changes are the result of variations in the yeast transcriptional program mainly controlled by the MAPK Slt2/Mpk1 through the cell wall integrity MAPK pathway (Arroyo et al., 2009; García et al., 2004; Levin, 2011).

The cell wall salvage response is very well conserved also in other fungi. Particularly the up-regulation of chitin synthesis as a consequence of cell wall stress has been observed in C. albicans (Walker et al., 2010) and A. fumigatus (Fortwendel et al., 2010; Meyer et al., 2007) and may be clinically relevant. In fact, C. albicans compensate echinocandin treatment, which inhibits β-1,3-glucan synthesis, by increasing cell wall chitin content (Walker et al., 2008) leading to a reduction in susceptibility to the drug. The majority of pathogenic Candida species display this compensatory chitin synthesis response as a potential mechanism of tolerance to caspofungin (Walker et al., 2013). Treatment of A. fumigatus with caspofungin or calcofluor white also increases cell wall chitin content, and up-regulation of chitin synthesis is dependent on AChsG (Walker et al., 2015). Moreover, transcriptional regulation of chitin synthases also controls paradoxical growth of A. fumigatus in response to caspofungin (Fortwendel et al., 2010). Therefore, compensatory reactions complicate the development of effective antifungal therapy using cell wall inhibitors.

Understanding the molecular basis of fungal adaptation through these mechanisms would offer the opportunity to circumvent these hindrances by interfering with them. The strengthening of the cell wall by overproduction of chitin suggests that covalent association of the glucan network to chitin, through the action of Crh transglycosylases, is also required for the compensatory mechanism. A more direct link between chitin–glucan cross-links, Crh function and cell wall stress resistance came out from studies analyzing these cross-links in yeast cell walls under heat stress. A temperature shift from 30 °C to 38 °C increased the proportion of chitin attached to β-1,6-glucan, as a consequence of the overexpression of CRH1 through the CWI pathway and relocation of both Crh1 and Crh2 to the lateral cell wall (Cabib et al., 2007). Moreover, glucan–chitin transglycosylation, measured by incorporation of SR-labeled glucan oligosaccharides in vivo clearly increases in a gas1Δ mutant in a Crh dependent manner (Cabib et al., 2008), as part of the chitin compensatory response in this mutant (Valdivieso et al., 2000). CRH genes are also transcriptionally induced in C. albicans (Liu et al., 2005) and Aspergillus (Meyer et al., 2007). Additionally, proteomic approaches have also identified in C. albicans an increase in the levels of a core of cell wall proteins, including β-1,3-glucan transglycosylases Phr1 and Phr2, the chitin–glucan trannglycosylases Chr11 and Utr2 and the cell wall maintenance protein Ecm33, in response to several surface-stress conditions, including growth at elevated temperatures (Heilmann et al., 2012).

All these data are in agreement with the induction of chitin–glucan transglycosylating activity as a general effective mechanism to cope with cell wall stress. A recent report by Ene et al. (2015) points to a new level of cell wall dynamism in C. albicans which permits major architectural changes within seconds of a hyperosmotic shock. Cell wall elasticity is increased by inactivation of the Crh cross-linking enzymes whereas overexpression of these proteins provides protection against hyperosmotic stress. Thus, the chitin–glucan enzymes of the CRH family modulate osmotic stress resistance in C. albicans (Ene et al., 2015).

Further supporting an important role for the Crh proteins to cope with cell wall stress, deletion of chitin–glucan transglycosylase encoding genes of the CRH family results in additive hypersensitivity to cell wall interfering compounds. In S. cerevisiae single deletion of CRH1 or CRH2 renders cells hypersensitive to Congo red, whereas simultaneous deletion of both genes results in a more severe phenotype of hypersensitivity (Cabib et al., 2007; Rodríguez-Peña et al., 2000). Similarly, deletion of genes of this family results in additive sensitivity to Congo red, calcofluor white or SDS in C. albicans (Pardini et al., 2006), whereas in A. fumigatus, the quintuple mutant is also hypersensitive to Congo red (Fang et al., 2015). Moreover, in yeast, deletion of these genes in strains deleted in genes important for cell wall biosynthesis or remodeling like FKS1, encoding for β-1,3-glucan synthase activity, or GAS1, encoding for a β-1,3-glucanosyl transferase, clearly aggravated their phenotypes (Cabib et al., 2007).

The discovery of novel antifungal drugs depends on the identification of unexplored targets. Although the fungal cell wall is theoretically an abundant source of target candidates for antifungal drugs (Latgé, 2007), the current cell wall specific antifungal arsenal applied into the clinics is limited to the family of echinocandins, including micafungin, anidulafungin and caspofungin (Tada et al., 2013). Chitin synthesis inhibitors like nikkomycins and polyoxins, both substrate analogs of UDP-N-acetylgalactosamine, are active in vitro but less effective in vivo (Zhang and Miller, 1999).

Besides chitin and glucan synthesis, cross-linking enzymatic activities conserved in the fungal kingdom, like those catalyzed by the Crh transglycosylases, are potential antifungal targets for new antifungal drug developments. These GPI anchored enzymes catalyze chitin–glucan transglycosylation in the periplasmic space. This is an advantage when looking for inhibitors because of their easier accessibility to the target (Amanianda and Latgé, 2010). Although these proteins are not essential for fungal viability, their enzymatic activities could be potential antifungal targets because cell wall is weakened as a consequence of their absence and, as stated above, they play an important role in the remodeling of the fungal cell walls necessary to counterbalance cell wall stress through compensatory mechanisms. Therefore, more-sophisticated approaches...
through combination therapies that synergistically target β-1,3-glucan synthesis and the cross-linking of chitin to β-1,3-glucan and β-1,6-glucan by the inhibition of the Crh activities can be envisioned as a possible effective strategy for the development of future successful antifungal therapies.

The development of the tools necessary for studying the cross-links between chitin and glucan both in vivo and in vitro, described throughout this paper, enables the identification of potential inhibitors of the transglycosylase activity mediated by the Crh proteins. Initial studies suggest that chitooligosaccharides behave as inhibitors of this activity both in vitro and in vivo. In vitro, incorporation of SR-labeled β-1,3-oligosaccharides into cell walls is inhibited by chitooligosaccharides (Cabib et al., 2008). In vivo, cla4Δ mutant cells growing in the presence of triacetetyl-chitotriose develop morphological defects similar to those found in a cla4Δ crh1Δ crh2Δ mutant strain (Blanco et al., 2012). Cla4 is a PAK kinase required for septin ring assembly in S. cerevisiae (Versele and Thore, 2004). Supporting the possibility of combination therapies, a partial synergistic effect on cell growth was found by simultaneous treatment of yeast cells in the presence of cell wall interfering compounds and triacetetyl-chitotriose (Sanz, A.B. and Arroyo, J., unpublished). The high-throughput assay method for transglycosylase activities recently developed by us (Kovácová and Farkas, 2016) offers the possibility for future large-scale screening of potential Crh inhibitors. Future identification of these inhibitors will allow to test their antifungal effect in combination with echinocandins and the validity of drug combination can be established.

Conclusions and future perspectives

The biosynthesis of individual fungal cell wall components has been largely characterized, especially in S. cerevisiae but also in C. albicans and A. fumigatus. However, the knowledge about enzymatic activities involved in the final phases of cell wall formation, specifically those required for cross-linking between cell wall polysaccharides was, until recently, much more limited. The work developed with S. cerevisiae in the last few years has allowed to learn essential aspects of the formation of linkages between chitin and glucan at the cell wall, the properties of Crh transglycosylases responsible of these cross-links and the biological functions that they exert in this organism. Because these enzymes are unique to fungi and very well conserved in most of the fungal genomes, including those of fungal pathogens, it will be interesting to functionally characterize orthologs in other organisms, particularly in filamentous fungi.

The characterization of fungal compensatory responses elicited under cell wall stress conditions open avenues for designing new antifungal treatment strategies. Current knowledge about the functional role of Crh proteins in cell wall remodeling under these circumstances highlights the potential of combination therapies targeting the synthesis of β-1,3-glucan and cross-linking between chitin and glucan by Crh inhibitors to prevent adaptive/compensatory mechanisms. Combination therapies using inhibitors of β-1,3-glucan synthesis with inhibitors of O-mannosyltransferases, N-mannosyltransferases, chitin synthesis, β-1,3-glucosyltransferases or inhibitors of Calcineurin, whose signaling route has been involved in regulation of fungal compensatory responses (Lagorce et al., 2003; Walker et al., 2008), has also been previously proposed and can be explored in the future as alternative antifungal treatments (Aimanianda and Latgé, 2010; Liu et al., 2015; Steinbach et al., 2007; Tada et al., 2013; Walker et al., 2010). In addition to susceptibility to antifungal agents, cell wall cross-links may also have implications in fungal virulence (Latgé and Beauvais, 2014). In agreement, cells of C. albicans deleted for Crh transglycosylases are avirulent in a mouse model of C. albicans systemic infection (Pardini et al., 2006). Moreover, A. fumigatus Crh orthologs mediates IL-17A sensing by fungi, a mechanism whereby fungi may adapt to their hosts ensuring their own persistence in an immunologically dynamic environment (Zelante et al., 2012).

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Conflict of interest

Authors disclose no potential sources of conflict of interest.

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