Published in final edited form as:

Yeast. 2010 August ; 27(8): 479–488. doi:10.1002/yea.1787.

On the evolution of fungal and yeast cell walls

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Abstract

Recent developments in genomics and proteomics provide evidence that yeast and other fungal cell walls share a common origin. The fibrous component of yeast cell walls usually consists of β -glucan and/or chitin. *N*-glycosylated proteins form an amorphous, cross-linking matrix as well as fibres on the outer surfaces of the walls. While the enzymes responsible for cross-linking walls into covalent complexes are conserved, the wall-resident proteins have diversified rapidly. These cell wall proteins are usually members of multi-gene families, and paralogues are often subject to gene silencing through epigenetic mechanisms and environmentally induced expression regulation. Comparative studies of protein sequences reveal that there has been fast sequence divergence of the *Saccharomyces* sexual agglutinins, potentially serving as a driver for yeast speciation. In addition, cell wall proteins show an unusually high content of tandem and non-tandem repeats, and a high frequency of changes in the number of repeats both among paralogues and among orthologues from conspecific strains. The rapid diversification and regulated expression of yeast cell wall proteins help yeast cells to respond to different stimuli and adapt them to diverse biotic and abiotic environments.

Keywords

cell wall evolution; yeast adhesins; gene family; tandem repeat; epigenetic regulation; adaptation

All three domains of life have organisms with cell walls, and in fact the walled state may be more common than unwalled [10,11,50]. The vast majority of fungi have walls, including all of the yeasts. The genomic and proteomic data recently made available and some new evolutionary analyses have rendered new insights into the evolutionary histories and processes of fungal and yeast cell walls. Here, we discuss the current state of our knowledge about the origin and evolution of yeast cell walls, using as our starting points the well-characterized walls of *Saccharomyces cerevisiae* and *Candida albicans*, and offer a more complete view of the evolution of yeast cell walls by synthesizing studies using a variety of approaches.

Evolutionary origin of fungal cell walls

In eukaryotes, cell walls are a diverse set of organelles, but in general they are composed of a fibrillar component (protein or polysaccharide) and a cross-linked amorphous matrix (usually glycoprotein) (Figure 1). Despite the apparent conservation of polymers in Figure 1, it is clear that there are major differences between wall structures in different lineages

[25,40]. As a matter of fact, plant and fungal cell walls are highly diverse in their polysaccharide, protein and other components.

For eukaryotic cell walls, the conservation of occurrence and diversity of compositions and architectures lead us to two hypotheses about their evolutionary origins: (a) that walls may have originated once but subsequently diversified in different evolutionary lineages; or (b) that walls have originated and evolved independently in different lineages. Recent evolutionary analyses of the cell wall biosynthetic and structural components have shed new lights on this question.

Fibrous wall polysaccharides

On the bases of comparative studies of cytology and life cycles, Cavalier-Smith has proposed that eukaryote cell walls are derived from chitinous cysts in the last common ancestor to the eukaryotes [10]. This proposal is reasonable, but has lacked systematic support until recently. A 2009 review has admirably covered the occurrence of key enzymes and glycoproteins in the fungi [47]. These reviewers concluded that the common ancestor of the opisthokonts (animals, fungi and choanozoa) had a chitin synthase, and that the first fungal ancestor also had a chitin deacetylase. In contrast, it is unclear whether β -glucan synthases were present ancestrally, as they are missing from microsporidia and chytrids, clades that diverged early in fungal evolution. However, fungal and plant β 1,3-glucan synthases are homologous, consistent with these enzymes being ancestral but being lost from early-branching lines in the fungi. For both the chitin and β -glucan pathways, gene duplications have led to divergence of paralogues of each of the key biosynthetic enzymes in many fungi.

Glycoproteins

The second major class of cell wall components is the glycoproteins (Figure 1). Cavalier-Smith has argued that *N*-glycosylation was a key evolutionary invention leading to the ability to replace rigid cell walls with flexible extracellular matrices. Flexible extracellular matrices would have enabled a phagotrophic lifestyle, leading to engulfment of other cells and consequent endosymbiotic relationships that were key to the emergence of eukaryotes [10,11].

N-linked glycoproteins are universal components of eukaryotic extracellular matrices and cell walls. The enzymes for standard *N*-linked oligosaccharide synthesis are almost universally present in the eukaryotes and archaea [49,55]. In the yeasts, glycoproteins have high mannose content and lack sialic acid and other 'outer chain' structures that characterize animal glycoproteins. However, there are few biochemical analyses of glycoproteins or genome sequences from fungi other than ascomycetes or basidiomycetes, so we do not yet know whether high mannose represents an ancestral state of fungi [47].

Functions and maintenance of cell walls

A variety of approaches have addressed the selective advantages that have retained walls in fungi. After all, walls represent ~25% of the dry weight of yeast cells, so they are metabolically expensive [38]. Resistance to viral infection is probably an ancient and potent selective pressure to maintain a cell wall [21]. In support of this idea, fungi appear to have unusually few viral pathogens, relative to other phyla. Walls also lead to survival after ingestion: there is a recent finding that chitinous cysts resist digestion in flies [13]. Although the last eukaryotic common ancestor was definitely not plagued by flies, the enzyme-resistant nature of chitin and chitosan would make them ideal coatings for dormant forms of organisms, rendering the cysts indigestible to amoebae and other protists, which are major predators in soil [54].

The evolution of fungal cell wall structure: theme and variations

The theme: walls in *S. cerevisiae* and *C. albicans*

With cautions about generality in mind, we discuss the evolution of wall structures using the model developed in *S. cerevisiae* and confirmed in *C. albicans* (Figure 2) [25,38]. There is a network of helical β 1,3-glucan fibres (structure 2). On average, each strand is transglycosylated to a molecule of branched β 1,6-glucan (structure 3). This branched glucan, in turn is transglycosylated with chitin (structure 1) and GPI-anchored proteins (structure 5). Chitin represents only a small percentage in *S. cerevisiae*, but the content is higher in other yeasts and filamentous fungi.

The wall glycoproteins (ellipsoids) are cross-linked to the polysaccharides to form a covalently linked complex. The known cross-linking structures are GPI-derived cross-links to β 1,6-glucan (structure 5), disulphide bonds (structure 8), glycoester bonds between deamidated Gln residues and hydroxyl groups on β 1,3-glucan (structure 4) and dityrosine cross-links (not shown) [38]. Wall glycoproteins can have any one or more of these covalent structures. The glycoproteins are uniformly *N*-glycosylated (structure 7) and/or *O*-glycosylated (structure 6) with high-mannose structures.

The variations: walls in other fungi

The generality of the above model cannot be directly confirmed or refuted, as no other fungus has had the depth of biochemical, genetic, and genomic analyses of *S. cerevisiae* and *C. albicans* walls. However, some known differences in structure include the lack of chitin in the vegetative cell walls of the fission yeast *Schizosaccharomyces pombe* and the missing of β 1,3-glucans in some basal fungi, although the latter is present in ascomycetes, basidiomycetes and the sporangiospore of *Mucoromycotina* (zygomycetes), as well as in plants and chromalveolates. The cross-linking polysaccharide β 1,6-glucan is present in ascomycetes, but its prevalence is currently uncertain in other fungal phyla, although it is present in chromalveolates. Furthermore, in some fungi, the major fibrous polysaccharide is α -glucan rather than β -glucan [25].

The evolution of cell wall-associated genes and proteins

The GO database category 'cell wall-associated' proteins (and their encoding genes) include those that make up cell walls, as well as those involved in the biosynthesis of cell walls. These two classes differ in functions, amino acid sequences, composition and evolutionary histories. In a study of 183 *S. cerevisiae* cell wall-associated genes (as determined from the GO annotation) and their paralogues, we found a high degree of conservation of cell wall biosynthetic and processing enzymes across 18 fungal genomes and in other eukaryote kingdoms [16,27]. Many of these proteins, mostly not actually located in the wall, have average amino acid composition of globular proteins, and are well aligned by standard techniques, and their evolutionary histories have been well characterized [47].

In contrast, proteins actually resident in yeast cell walls are much less conserved and thus will be the focus of the following discussions on the evolution of wall-associated proteins. These wall-resident proteins include cell wall structural proteins, adhesins and invasins [19], carbohydrate-active enzymes (glycosidases and transglycosylases), osmotic stress sensors, proteases and iron transport proteins [25]. These proteins are mosaics of functional globular domains (usually in the *N*-terminal region) and regions with low amino acid complexity and many repeats, either tandem or dispersed (Figure 3) [14,15,25]. The globular domains are well conserved and usually present in families of paralogous genes [47], while the other regions of the proteins show signs of rapid evolution. However, different groups of wall-resident proteins also show dramatically different patterns of evolution, as illustrated below.

Conservation of cross-linking enzymes

Carbohydrate-active enzymes (transglycosylases) are responsible for cross-linking walls into covalent complexes. The *CRH* family transglycosylases cross-link chitin to β -glucans and are conserved in at least ascomycetes and basidiomycetes [6,7,16]. The *GAS/GEL/PHR* family of glucan transglycosylases is present in ascomycetes and basidiomycetes, and the X-ray structure of a catalytic region demonstrates that these domains are well-structured and conserved [2].

Other key enzymes in wall biogenesis and remodelling are less well characterized. The enzymes that cleave GPI glycans and transglycosylate to β 1,6-glucan acceptors are not known, but the Dfg5 and Dcw1 paralogues are likely candidates [26,36,37]. These enzymes are present in both ascomycetes and basidiomycetes [27]. However, proteomic analyses have failed to find glucan-linked GPIs in *Aspergillus fumigatus*, an ascomycete, and *Ustilago maydis*, a basidiomycete [27,47,59]. Currently, we have no information on the enzymes responsible for the formation of the protein-glycoesters, but proteomic analyses uniformly report that mild base treatment can extract proteins from cell walls, consistent with the presence of ester cross-links. The characteristic Pir sequence repeats that participate in these bonds (Q[IV]XDGQ[IVP]Q) have been found only in yeasts closely related to *S. cerevisiae*; other protein sequences must serve as carboxyl donors in other fungi. Therefore, the prevalence and evolution of such linkages are currently unknown. Similarly, we have no information on the enzymes responsible for extracellular disulphide formation.

The diversity and controlled expression of yeast cell surface proteins

On the outmost layer of yeast cell walls there are often glycoprotein fibrils [8,38]. Just like the extracellular matrices in animals and plants, the extracellular proteins forming this outmost layer in yeasts play pivotal roles in contact and communication with the environment, both biotic and abiotic. Changes in the yeast cell surface proteins alter the ability of yeast cells to bind to other yeast cells for mating reactions, mat and biofilm formation or, in the case of pathogenic yeasts, to host tissue and indwelling medical devices. These proteins are thus often collectively called adhesins. Changes in yeast cells' adhesion ability are of significant biological, industrial and medical relevance [19]. New genomic data and comparative analyses suggest that these proteins show especially fast evolution, driving the diversification of yeasts and adapting them to various environments.

Basic structure of yeast adhesins

The saccharomycetous yeast adhesins have a common sequence of domain types in their ORFs. An *N*-terminal secretion signal is generally followed by globular binding domains, often Ig- or lectin-like; Thr-rich tandem repeats, which also have binding activity; then a highly glycosylated Thr-Ser-rich stalk region that extends the binding domains into the intercellular medium. The adhesins are anchored to cell wall glucan through modified GPI anchors [19,38]. In the ascomycete fission yeast *Sz. pombe*, the order of domains in the adhesin ORFs are different and GPI anchors are absent, but the amino acid compositions and sequence characteristics of the individual domains are similar [42,51]. Interestingly, yeast adhesins are particularly rich in long tandem repeats. A recent bioinformatic study found that 18 of 29 ORFs (62%) containing long (>40 nt) tandem repeats in *S. cerevisiae* genome encode cell surface proteins while the latter makes only 1.3% of all *S. cerevisiae* ORFs (88 of 6591) [57].

Sexual adhesin function and evolution

The *S. cerevisiae* sexual agglutinins facilitate the mating between two types of cells, **a** and **α** (Figure 4). **a**-Agglutinin consists of two subunits, encoded by two unlinked genes, *AGA1*

and *AGA2*. The *Aga1p* subunit functions as the anchor of **a**-agglutinin. However, it is expressed in both mating types. In contrast, the *Aga2p* subunit is expressed only on **a** cells and gives **a**-agglutinin its binding specificity. The adhesin *Fig2p* is a binding partner of *Aga1p*, and both proteins have homologous low complexity and repeat sequences important in adhesion and mating [60]. The complementary adhesin **α** -agglutinin is encoded by a single gene, *SAG1*, and contains three Ig-like domains and a Ser–Thr-rich stalk region.

Studies of protein and nucleotide sequences of the sexual adhesins showed similar rates of synonymous nucleotide substitutions (codon changes without changing amino acids) but a much higher rate of non-synonymous substitutions (codon changes resulting in amino acid replacements) than any other group of cell wall proteins. The between-species diversification for them is extensive, while the sequences are highly conserved within species [Xie *et al.*, in preparation]. Thus, they could have been a driving force in yeast speciation.

Aggregative adhesins

Other adhesins mediate interactions between yeast cells to form aggregates leading to mats and biofilms. In general, these adhesins are members of multigene families, with individual paralogues expressed under different environmental stimuli [25,38]. The *S. cerevisiae* *FLO* family genes and *MUC1* (also called *FLO11*) encode mannose-specific lectins that cause spontaneous aggregation or flocculation. This property has been exploited in brewing to separate the yeast from the product. *Flo1p* mediates mat formation and is the first known ‘green-beard gene’, which gives an evolutionary advantage to altruistic behaviour under stressful conditions [53].

The paralogous genes *FLO1*, *FLO5*, *FLO9* and *FLO10* are subject to epigenetic silencing because of their subtelomeric locations. This is likely due to the fact that subtelomeric regions are rich in repetitive sequences, including transposable elements and non-coding tandem repeats, which are often epigenetically silenced and consequently silence neighbouring genes by DNA methylation and/or chromatin modification. However, these normally silenced genes can be activated by mutations in other genes. For example, *FLO10* is expressed and produces hyperfilamentation and hyperadhesion in *ira* mutants, although the expression of the *FLO10* gene is epigenetically unstable and switches between ‘on’ and ‘off’ at a very high frequency [30].

In contrast, *MUC1* is neither centromeric nor telomeric and is expressed in many laboratory strains of *S. cerevisiae*, where it exhibits a variety of phenotypes. Its expression is required for pseudohyphal and invasive growth, considered adaptive responses to starvation for nitrogen because they allow yeast to forage for nutrients [19,24]. Nutritional signals such as nitrogen starvation induce the expression of *MUC1* in *S. cerevisiae* through both the mitogen-activated protein kinase and protein kinase A pathways [48]. However, even under the inductive nutritional condition, some individual cells express *MUC1* while others do not, an observation implying reversible epigenetic repression by chromatin-binding proteins [30].

Adhesins in host–pathogen binding

Adhesins from the opportunistic pathogens *C. albicans* and *Candida glabrata* are also well studied. With eight members, the *C. albicans* *ALS* gene family is orthologous to *S. cerevisiae* *SAG1*. *ALS* adhesins bind peptide ligands and thus mediate many activities, including adherence to human epithelia cells and iron acquisition [1,43]. *Als* proteins have Thr-rich tandem repeats that mediate yeast aggregation and adherence to artificial surfaces to mediate biofilm formation. The repeats vary widely in number among different *ALS*

genes, but have conserved sequences [23,44,61]. In *C. glabrata*, the *EPA* gene family contains 17 members and encodes adhesins mediating binding to host epithelia during infections [17,64]. Epa proteins are also involved in aggregation of the *C. glabrata* cells to form either microscopic colonies or biofilm [35,56]. The *C. albicans* Hwp adhesins also participate in biofilm formation [43]. Another adhesin, Eap1p, mediates the stable binding of *C. albicans* to plastic surfaces, such as those of indwelling catheters and other medical devices, and thus may be critical in prosthesis-induced candidaemias and endocarditis [41].

The members of *ALS* family are expressed at different stages of growth and infection and associated with different morphological forms in *C. albicans* [28,32]. Laboratory experiments show that *ALS1* is maximally transcribed in response to growth-medium components [34]. The expression of *ALS3*, however, is hypha-specific [33]. Some studies have revealed transcription from all *ALS* genes during *C. albicans* pathogenesis; however, different members were observed to be expressed at different levels, with *ALS1*, *ALS3* and *ALS9* being detected most frequently [12,28,29].

In *C. glabrata*, most members of *EPA* gene family are located in subtelomeric regions and subject to histone deacetylase *SIR*-dependent transcriptional silencing. Two *EPA* genes, *EPA6* and *EPA7*, are normally silent but are transcribed and contribute significantly to the adhesion of the mutant cell with *SIR3* deletion [9]. The expression of normally silent *EPA* genes can be induced by the limitation of nicotinic acid, a precursor of NAD^+ , and the induced expression is likely the result of a reduction in NAD^+ availability for the NAD^+ -dependent histone deacetylase Sir2p [18]. All these evidences suggest an epigenetic mechanism by chromatin modification regulating *EPA* gene expression.

Adaptive evolution of yeast cell wall genes and proteins

Thus, proteins resident in cell walls show key characteristics that promote rapid evolution: paralogous gene families; both tandem and dispersed repeats; and, in the case of the sexual adhesins, a high rate of amino acid replacements between species. As demonstrated above, paralogy leads to functional divergence and adaptive expression patterns through genetic and epigenetic regulatory mechanisms [17,18,22,32]. Some members of the same adhesin gene family are often closely linked or even exist in tandem. For example, the *EPA1*, *EPA2* and *EPA3* genes are very closely linked on the same chromosome in *C. glabrata*, while the *ALS1*, *ALS5* and *ALS9* genes are arranged in tandem on chromosome 6 of *C. albicans*. This pattern suggests tandem gene duplication as a possible mechanism for the expansion of adhesin gene families. The sequence similarity between adhesin genes family members, either closely linked or on different chromosomes, induces recombination, which in turn could create partial or whole-gene duplication or deletion, hybrid alleles or chimeric genes of new functions. Indeed, *C. albicans* strains lacking either the *ALS1* or the *ALS5* gene have been described [62,63]. Similarly, in *S. cerevisiae*, *FLO1*, *FLO5* and *FLO9* genes have also been found to have adjacent, truncated, non-functional pseudogene copies, probably the relics of unequal crossover events [58]. These pseudogene copies in turn could recombine among themselves or with functional members of the same family to form new chimeric genes [39,19].

The richness of tandem or dispersed repeats in the coding region of yeast adhesins also increases the rate of recombination between orthologous alleles and paralogous genes. Unequal crossing-over or gene conversion from homologous recombination between alleles during meiosis and/or the ‘illegal’ recombination between paralogous gene family members, together with DNA replication slippage, create expansion or contraction of the tandem repeat region. Consequently, the number of repeats often differs, not only among members of the same gene families but also among conspecific strains for the orthologous genes. The

ALS, *EPA* and *FLO* families have all shown a great range of variation in the sequences and number of tandem repeats among paralogous gene members [32,58; Xie *et al.*, in preparation]. A study of *ALS7* identified 60 alleles in 66 strains of *C. albicans*, largely caused by the rearrangements of repeat elements [61]. A large-scale study of adhesins in *S. cerevisiae* and its most closely related species, *S. paradoxus*, also reveals tremendous variation within both species as well as differentiation between the two species in the tandem repeat regions [Xie *et al.*, in preparation].

Ab initio modelling of the tandem repeat sequences in Als proteins consistently revealed antiparallel β -sheet structures that serve important functions [23]. Experimental evidence shows that the tandem repeats in Als5p greatly increase cell-to-cell aggregation in *C. albicans* [45] and Als proteins with more copies of the tandem repeats are more aggregative [31,44,45]. So the tremendous variation in the tandem repeat regions in yeast adhesins could potentially generate great diversity in their functions, which could adapt yeast cells to changing or new environments.

Adaptive features displayed by yeast adhesins, including the richness in repeats and low-complexity sequences, prevalence of paralogous gene families and predominantly subtelomeric locations, are shared by cell surface proteins in other organisms [40,56]. For example, *Trypanosoma cruzi*, the agent causing Chagas' disease, contains 844 members of the subtelomeric *TcMUC* subfamily of mucins, which are associated with adhesion and attachment to mammalian host cells. Members of this subfamily show strong allelic variation, particularly in the number of repeats in their coding regions [3,5,20]. Furthermore, a change in mucin expression accompanies the transition from the insect host to mammalian host for this parasite [3,5]. In malaria-causing *Plasmodium falciparum*, the erythrocyte membrane protein 1 (PfEMP1) confers adhesion to host tissues and blood cells and is encoded by about 60 *var* genes, some of which are clustered at telomeres. Frequent recombination events among *var* genes generate great protein diversity on the cell surface. The variable expression of *var* genes has been documented to help *P. falciparum* to evade the host immune system for successful colonization [4,46,52].

Conclusions on the origin and evolution of yeast cell walls

Therefore, the evidence supports the idea that fungal cell walls are homologous structures derived from an ancestral wall made of chitin and perhaps β -glucan and cross-linked through N-glycosylated glycoproteins. Nevertheless, there is unusual structural and functional diversity in cell wall proteins and their encoding genes. Yeast cell wall proteins are particularly enriched in tandem repeats, which vary greatly in number, even among orthologous gene copies from different strains of the same species, as well as in sequences among paralogous family members. Mutations in such repeats can generate broad functional diversity, adapting yeasts to varying environments or facilitating their exploration of new ones [56,64]. As a special group of cell surface proteins, yeast sex adhesins are largely conserved within species but show high levels of amino acid replacements between species, suggesting an essential role in driving yeast speciation, or the formation of new species.

Acknowledgments

We thank Dr Weigang Qiu, Dr Susan Epstein and Dr Saad Mneimneh for comments and discussions. We also would like to thank the two anonymous reviewers for their comments in improving the manuscript. Work in the authors' laboratory was supported by NIH SCORE Program Grant No. SC1 GM083756.

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Figure 1.

Some representative organisms and the major components of their cell walls or extracellular matrices. Four representative eukaryote kingdoms are: 1, fungi; 2, animals; 3, amoebazoa; and 4, plants. The fungal phyla are: A, ascomycetes; B, basidiomycetes; and C, microsporidia

Figure 2.

Cartoon model of the cell wall of an ascomycetous yeast. The general arrangement includes polysaccharides (lines) and glycoproteins (ellipsoids). The specific structures are: 1, chitin; 2, β 1,3-glucan; 3, β 1,6-glucan; 4, glycoprotein–glutamyl–glycoester bonds; 5, glycoprotein GPI-glycan remnant linked to β 1,6-glucan; 6, glycoprotein O -glycan chain; 7, glycoprotein N -glycan chain; and 8, disulphide bond. Reprinted with permission of Elsevier from Gonzalez *et al.* (2009) [25]

Figure 3.

Fractions of cell wall protein sequences that are in motifs (tandem or dispersed repeats), grouped by decile. The total length of the repeated elements found in each cell wall protein was divided by the length of the protein. Reprinted with permission from Coronado *et al.* 2007 [15]

Figure 4.

Expression and interactions of sexual adhesins in *S. cerevisiae*. Double-headed arrows denote the interactions among the adhesins [19,60]. Each of these genes has a high rate of non-synonymous substitutions between *Saccharomyces* species