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1 **Involvement of amyloid proteins in the formation of biofilms in the**
2 **pathogenic yeast *Candida albicans*.**

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25

26 **Abstract**

27

28 *Candida* species represent a major fungal threat for human health. Within the *Candida*
29 genus, the yeast *Candida albicans* is the most frequently incriminated species during episodes
30 of candidiasis or candidemia. Biofilm formation is used by *C. albicans* to produce a microbial
31 community that is important in an infectious context. The cell wall, the most superficial
32 cellular compartment, is of paramount importance regarding the establishment of biofilms. *C.*
33 *albicans* cell wall contains proteins with amyloid properties that are necessary for biofilm
34 formation due to their adhesion properties. This review focuses on these amyloid proteins
35 during biofilm formation in the yeast *C. albicans*.

36

37 **Keywords:** Human fungal pathogen; *Candida albicans*; biofilm; amyloid proteins; adhesion;
38 cell wall.

39

40 **Introduction**

41 Human fungal pathogens are frequently encountered in superficial infections of the skin
42 and the nails. These infections affect nearly 1 billion people worldwide and do not pose a
43 threat for patient survival [1]. However, in certain circumstances fungi can invade the
44 bloodstream and cause fungemia [2]. Among all the fungi responsible of fungemia, the
45 *Candida* genus is the most often incriminated [3]. Candidemia are bloodstream infections
46 caused by *Candida* species, and 54% of them are linked to *C. albicans* [3]. Although *C.*
47 *albicans* is a normal resident of the genital and gastro-intestinal tracts, it can move towards
48 the blood compartment in immunocompromised patients or patients suffering from dysbiosis
49 [4]. *C. albicans* is thus a tremendous burden on public health systems by being associated to
50 high mortality rates (> 40%), and high healthcare costs. Over the last decades the health

51 situation has worsened and the number of patients suffering from candidemia has raised
52 dramatically [5]. Misuse of antibiotics, increased resistance to antifungal drugs, unreliable
53 diagnosis and invasive care procedures are all the reasons that account for the increase of *C.*
54 *albicans*-based candidemia [6,7]. One of the major virulence traits in *C. albicans* is its ability
55 to alternate between the yeast and the hyphae morphotypes [8]. The yeast form is more
56 suitable for dissemination within the body via the bloodstream and adhesion to surfaces while
57 the hyphal form is required to cross epithelial barriers and to establish biofilms [8].
58 Regulation of biofilm formation has been addressed in several studies; thus, the molecular
59 mechanisms that govern biofilm formation at the transcriptional level within the nucleus are
60 rather well understood [9]. However, the involvement of the cell wall in biofilm development
61 is more nebulous. The purpose of this mini-review is to present the latest findings related to
62 the cell wall during biofilm formation or maintenance, with a special focus on amyloid
63 proteins.

64

65 **Biofilm formation in *C. albicans***

66

67 A biofilm can be defined as a highly structured three-dimensional community of microbial
68 cells that are adhered to biotic or abiotic surfaces and encased in an extracellular matrix
69 [10,11]. Biofilms are of tremendous importance for *C. albicans* pathogenesis because, in
70 addition to their adhesion properties, they decrease the efficiency of antifungal drugs as well
71 as the recognition by the immune system [10,12]. In biofilms, *C. albicans* can be found either
72 alone as a monomicrobial structure or associated to bacteria (e.g. *Staphylococcus aureus*,
73 *Streptococcus gordonii*, *Pseudomonas aeruginosa*) in a polymicrobial community [10,13].
74 Biofilm formation in *C. albicans* consists of four successive phases, namely adherence,
75 initiation, maturation and dispersion [14]. Adherence is the step during which round yeast

76 cells stick to a surface, creating a basal layer whose physiological function is to anchor the
77 biofilm to its substrate [11,14]. Then, the initiation step consists of cell proliferation and the
78 emergence of pseudo-hyphae and hyphae from adherent cells, a process called filamentation.
79 In the maturation phase, *C. albicans* cells continue to proliferate and to elongate to constitute
80 an intricate and thick network of yeast cells, pseudo-hyphae and hyphae. In addition, this
81 phase is characterized by the synthesis of an abundant extracellular matrix composed of
82 carbohydrates, lipids and nucleic acids [11,14,15]. This extracellular matrix will encase the
83 fungal cells and provide a physical barrier that protects the biofilm from physical insults from
84 the environment. Lastly in the dispersion phase yeast cells leave the biofilm to colonize
85 secondary infection sites in the human body [11,14]. The establishment of a biofilm is a
86 complex biological process used by *C. albicans* to adapt to physical and chemical changes in
87 its environment. As *C. albicans* is a normal resident of the human body, the environmental
88 cues that trigger biofilm formation are linked to human physiology. Indeed, increasing
89 temperature, presence of bacterial muramyl peptide or of N-acetyl glucosamine, CO₂ levels,
90 nutrient starvation and surface contact are stimuli that are recognized by the sensing systems
91 of *C. albicans* to initiate hyphal differentiation [16]. To date, numerous signaling pathways
92 have been described in the literature. Among them, the cAMP-PKA and the mitogen-activated
93 protein kinase (MAPK) are the 2 major signaling pathways that link the extracellular medium
94 and the transcriptional response orchestrated by the yeast to trigger the filamentation [16–18].
95 Regarding the formation of biofilm in *C. albicans*, numerous transcriptional regulators have
96 been linked to this biological process in the last decade [19]. Actually, genetic screens
97 performed on a collection of *C. albicans* single deletion mutants identified a “core” of nine
98 master regulators which are required to put in place biofilms in *in vitro* (polystyrene plates,
99 silicone squares) and *in vivo* (rat catheter, rat denture) models [9,19]. The transcription factors
100 Efg1, Bcr1, Ndt80, Tec1, Rfx2, Gal4, Flo8, Brg1 and Rob1 constitute this “core” of master

101 regulators and they coordinate the expression of approximately 1,000 genes [9,19]. The
102 majority of the genes that belong to this transcriptional network still lack a molecular
103 function, but some of them are correlated to biological processes required for biofilm
104 formation such as lipid metabolism (*EHT1*), regulation of filamentation (*ORF19.4000*) or cell
105 adhesion (*ALS1*, *HWPI*) [19,20]. Alongside the nine “core” transcription factors required for
106 biofilm formation, other relevant regulators have been identified. The transcription factor
107 Ume6, a downstream target of Efg1, can enhance biofilm formation by activating the
108 transcription of Hgc1 (control of hyphal development) and Sun41 (cell wall integrity) [21].
109 Strikingly, numerous genes that are regulated by this network encode proteins found in the
110 cell wall. Hence, the cell wall compartment is extremely important by allowing *C. albicans* to
111 assemble in the form of a biofilm.

112

113 **The cell wall of *C. albicans***

114

115 As a member of the fungi taxon, *C. albicans* owns a specialized cellular compartment
116 called the cell wall. Because this organelle is the first point of contact between *C. albicans*
117 and the host, it is crucial for fungus-host interactions [22]. Furthermore, the cell wall is
118 essential for *C. albicans* development by assuming physiological roles such as
119 morphogenesis, adherence, biofilm formation, immune system evasion, turgor, cell shape, cell
120 cycle and resistance to physical insults and chemicals [23,24]. *C. albicans* cell wall exhibits a
121 complex molecular architecture in the form of a multilayer structure. The innermost layer,
122 which is in direct contact with the plasma membrane, is rich in chitin molecules and covered
123 by a molecular assembly of β -(1,3)- and β -(1,6)-glucans. Molecules of chitin and β -glucans
124 interact with each other through hydrogen bonds to create an intertwined fiber network called
125 the inner cell wall [25]. The outer cell wall corresponds to the second layer of this cellular

126 compartment and it consists exclusively of highly mannosylated glycoproteins [25]. These
127 glycosylated proteins belong to several classes of proteins such as adhesins (adhesion),
128 yapsins (protease activity for cell wall remodeling), hydrolases and deacetylases (cell wall
129 plasticity) [25]. The cell wall is a highly dynamic organelle whose architecture is reshaped
130 during the transition between yeast and hyphae morphotypes and according to variation of the
131 environment [26,27]. Indeed, FITR spectroscopy experiments have revealed that *C. albicans*
132 hyphae exhibits 10% more β -1,3-glucans and 20% less β -1,6-glucans than yeast-shaped cells
133 [28], and their structure also varies in hyphae as compared to yeast cells [29]. Under the
134 hyphal form, β -glucans adopt a cyclical structure where the β -1,3-linked polymer backbone is
135 decorated with β -1,6-linked side chains [30]. The molecular mechanisms underlying the cell
136 wall remodeling during the transition from the yeast form to the hyphal form are still largely
137 unknown. However, the β -1,3-glucanoyltransferase Phr1 and the β -glucanase Mp65 are
138 increasingly being thought to reshape the cell wall in response to environmental cues that
139 trigger the filamentation process [31–33]. The glycoprotein pattern of the outer layer of the
140 cell wall is also modified when cells trigger the hyphal growth to form biofilms. The
141 reorganization of cell surface proteins is essential to sustain biological functions required for
142 biofilm development such as immune system escape or adhesion [34]. Furthermore, adhesins
143 from the Als family or the adhesin Eap1 are identified at the surface of *C. albicans* grown
144 under the hyphal mode [35]. One striking feature of these adhesins is that they display
145 amyloid properties [36].

146

147 **Relevant amyloid proteins used by *C. albicans* to develop biofilm**

148

149 Amyloids represent structured protein aggregates that are found in all kingdoms of life.
150 More specifically, amyloids adopt an unbranched and elongated high molecular weight

151 fibrillar form [37]. At the structural level, amyloids adopt a quaternary folding formed by the
152 assembly of multiple copies from the same protein [38]. Each of the monomers is organized
153 perpendicularly to the fibril axis by constituting a cross β -type molecular architecture [39,40].
154 For detailed description of the amyloid fibril structure see reviews from Eisenberg and
155 Sawaya as well as Close *et al.* [39,40]. Aggregates formed by amyloid proteins have long
156 been considered harmful for biological systems [41]. However, the concept of functional
157 amyloid, where aggregated proteins play biological roles, has recently emerged [42]. A well
158 described example in the literature concerns the protein Cdc19 from *S. cerevisiae*. In the
159 presence of glucose, the pyruvate kinase Cdc19 is located in the cytoplasm to ensure the last
160 enzymatic reaction of glycolysis [43]. Conversely, following glucose starvation, Cdc19 self
161 assembles under the form of an amyloid-like aggregate before being sent within the P-bodies
162 [43]. This intracellular traffic of Cdc19 avoids its degradation under stressful conditions and
163 will improve the fitness of the cells during the following recovery phase [43]. *C. albicans*
164 proteins with amyloid properties have also been described in the literature. The majority of
165 these polypeptides are classified into the ALS-type family of adhesins [28,44]. All the Als
166 proteins share a common organization [45]; indeed, they all own a N-terminal Ig-like domain,
167 which gives them adhesive properties during attachment to surfaces (Fig. 1). Beside the Ig-
168 like domain, the Thr-rich T domain holds the amyloid forming sequence that is required to
169 trigger the assembly of the amyloid structures within the cell wall under specific conditions
170 (Fig. 1). In the middle part of the proteins, the TR domain is characterized by the presence of
171 tandem repeat sequences that allow hydrophobic interactions between adhesins on the one
172 hand and surfaces on the other hand (Fig. 1). At their C-terminal domain, Als proteins have a
173 highly glycosylated and Ser-Thr-rich region called the stalk, followed by a GPI anchor that,
174 upon maturation, serves to link adhesins to the cell wall glucans through glycosidic bonds
175 (Fig. 1). Among the Als proteins, only Als1, Als3 and Als5 have been shown to display

176 adhesion activity [28,44]. When Als1, Als3 or Als5 adhesins are subjected to shear stresses,
177 like those encountered in the blood flow within the human body, they have the ability to
178 organize themselves into nanodomains at the cell surface [27,46–49]. Consequently, Als1,
179 Als3 or Als5 are self-aggregated through the amyloid forming sequence supplied by each of
180 the monomers. Thus, the amyloid structures are crafted by the assembly of adhesins to
181 constitute the nanodomains, which will allow the creation of hydrophobic patches in specific
182 area of the cell wall and promote cell adhesion on biotic or abiotic surfaces [27,47]. It is
183 suggested that the adhesive properties of nanodomains are required to form biofilms, because
184 the lack of either Als1, Als3 or Als5 greatly reduced the ability of *C. albicans* to develop
185 biofilm. In addition to adhesins, the secreted aspartic peptidase Sap6 is also suspected to have
186 a function in the establishment of *C. albicans* biofilm [50]. Under its globular form, Sap6
187 displays a protease activity required for the virulence of the fungus. However, like Als
188 proteins, Sap6 contains amyloid-forming sequences in its primary amino acids structure [50]
189 (Fig. 1). It is thus suggested that Sap6 could adopt an amyloid shape that favors cell-cell
190 adhesion during biofilm formation [50]. Rbt1 is another protein of *C. albicans* that contains
191 amyloid forming sequences [51] (Fig. 1). In addition to the Flo11 domain (homotypic
192 interactions) and the serine/threonine-rich region, Rbt1 owns the conserved flocculin type 3
193 repeat that includes 2 amyloid-forming regions with high β -aggregation potentials [51] (Fig.
194 1). Physiologically, Rbt1 is located within the cell wall of *C. albicans* and positively affects
195 the formation of biofilm. Indeed, overexpression of the *RBT1* gene results in an increased
196 biofilm biomass as compared to a strain that does not overexpress it. The results that emerge
197 from this work on Rbt1 suggest that 2 amyloid-forming sequences with high β -aggregation
198 potential are involved in cell-cell interactions by fostering hyphae aggregation. In addition to
199 the last-mentioned proteins, Eap1 is a cell surface protein found in *C. albicans* that is able to
200 self-aggregate and form an amyloid fibre *in vitro* [37]. Eap1 is an adhesin involved in the

201 adhesion process of *C. albicans* on polystyrene as well as on epithelial cells [52]. However, to
202 date, there is no evidence that Eap1 could form patches at the cell surface as described for the
203 Als proteins [53]. Hence, the involvement of Eap1 as an amyloid in *C. albicans* biofilm
204 formation is purely speculative. Except for Als proteins, Sap6, Rbt1 and Eap1, knowledge on
205 the involvement of amyloid proteins during biofilm development is scarce. However, other
206 cell wall-associated proteins, namely Pga59 and Pga62, exhibit interesting features and might
207 also be involved in biofilm formation as amyloids. First, Pga59 and Pga62 are small proteins
208 that contain 3 and 6 predicted amyloid-forming sequences, respectively (Fig. 1). The presence
209 of these sequences strongly suggests that both Pga59 and Pga62 are able to organize
210 themselves in the form of amyloid structures. Second, loss of *PGA59* and/or *PGA62* has a
211 significant impact on cell wall integrity in *C. albicans*. Indeed, their concomitant inactivation
212 results in an increased sensitivity to cell wall-perturbing agents [54]. Further analyses using
213 mass spectrometry and electron microscopy also revealed that the absence of both Pga59 and
214 Pga62 disturbs the cell wall composition and its architecture [10,54]. Thirdly, overexpression
215 of *PGA59* has a positive impact on biofilm formation and cell adhesion [10]. Altogether, these
216 results led us to assume that Pga59 and Pga62 could be functional amyloids in *C. albicans*.
217 More precisely, the globular form of Pga59 would be involved in cell wall integrity and
218 architecture, whereas the suspected amyloid form of Pga59 would contribute to a yet
219 unknown adhesion mechanism during biofilm establishment. Overall, the role of amyloid
220 proteins in *C. albicans* biofilm formation is increasingly evident. Indeed, adhesive properties
221 of cell wall amyloid proteins (either through the Als nanodomains and/or by the action of
222 Sap6 and Rbt1) are used to build up *C. albicans* biofilms. However, how amyloid proteins are
223 used by *C. albicans* to develop biofilms is still poorly understood at the molecular level. The
224 study of proteins such as members of the Als protein family, Sap6, as well as Pga59 and

225 Pga62 if they prove to be genuine amyloid proteins, will allow us to deepen the knowledge
226 linking amyloid proteins and biofilm formation.

227

228

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237

238 **Conflict of interest.** The authors declare that they have no conflict of interest.

239

240

241 **Figure Legend**

242 **Fig. 1** Topological comparison of cell surface amyloid proteins in *C. albicans*. *A*, Typical

243 domains of the Als proteins family: Ig-like domain (blue), T domain (orange), TR domain

244 (red), Stalk (green) and GPI anchor (black). Amyloid-forming regions are represented as

245 purple circles. *B*, the secreted aspartic protease Sap6 is shown with its four amyloid-forming

246 regions (purple circles) and the integrin-binding (RGD) domain (yellow). *C*, Topological

247 organization of the protein Rbt1. The Flo11 conserved domain is shown in black while the

248 serine/threonine-rich region is colored in brown. The pink colored rectangle contains 2

249 amyloid-forming regions (purple circles) and corresponds to the conserved flocculin type 3

250 repeat. The GPI anchor is represented by a vertical black line. *D*, both Pga59 and Pga62 are
251 represented with their respective amyloid-forming regions (purple circles) and GPI anchors
252 (black lines). Protein length is indicated with numbers of amino acid residues under the sketch
253 of each proteins.

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255

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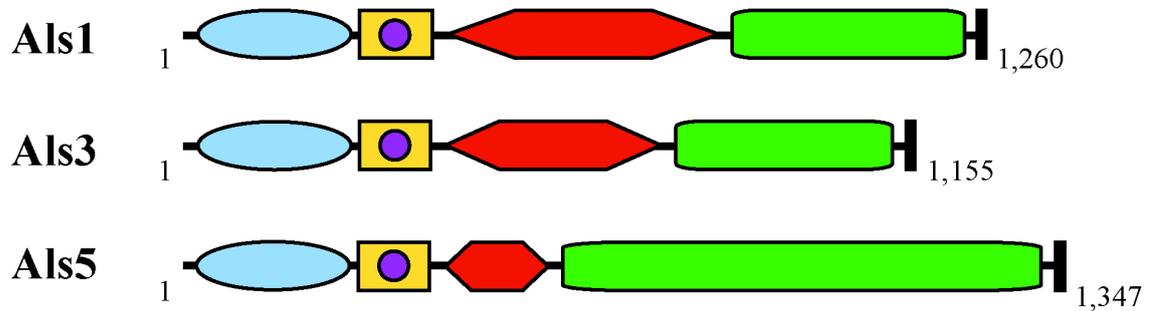
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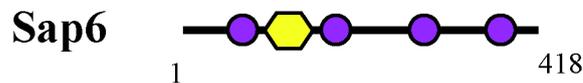
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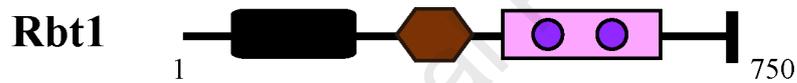
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