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The Als3-mediated attachment of enolase on the surface of *Candida albicans* cells regulates their interactions with host proteins

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Key Words:	enolase, agglutinin-like sequence protein Als3, moonlighting proteins, <i>Candida albicans</i> , plasminogen, kininogen

**The Als3-mediated attachment of enolase on the surface of *Candida albicans* cells
regulates their interactions with host proteins**

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Running title: Als3-mediated surface location of *C. albicans* Eno1

Summary

The multifunctional protein enolase has repeatedly been identified at the surface of numerous cell types, including a variety of pathogenic microorganisms. In *Candida albicans*—one of the most common fungal pathogens in humans—a surface-exposed enolase form has been previously demonstrated to play an important role in candidal pathogenicity. In our current study, the presence of enolase at the fungal cell surface under different growth conditions was summarized and a higher abundance of enolase at the surface of *C. albicans* hyphal forms compared to yeast-like cells was found. Affinity chromatography and chemical cross-linking indicated a member of the agglutinin-like sequence protein family—Als3—as an important potential partner required for the surface display of enolase. Analysis of *Saccharomyces cerevisiae* cells overexpressing Als3 with site-specific deletions showed that the Ig-like N-terminal region of Als3 (aa 166-225; aa 218-285; aa 270-305; aa 277-286) and central repeat domain (aa 434–830) are essential for the interaction of this adhesin with enolase. In addition, binding between enolase and Als3 influenced subsequent docking of host plasma proteins—high molecular mass kininogen and plasminogen—on the candidal cell surface, thus supporting the hypothesis that *C. albicans* can modulate plasma proteolytic cascades to affect homeostasis within the host and propagate inflammation during infection.

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4 **1. Introduction**
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7 Yeast-like fungus *Candida albicans* is among the common commensal colonizers of the
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9 human skin, vagina, oral cavity and gut, but can also initiate the development of a wide
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11 range of diseases in patients with impaired immunity. There is currently a broad clinical
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13 spectrum of these disorders, candidiases, ranging from mucocutaneous overgrowth to
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15 disseminated infections like candidemia (Lim, Rosli, Seow, Chong, 2012). The pathogenesis
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17 of *C. albicans* infections is mediated by several virulence factors and mechanisms,
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19 including the morphological transition from yeast-like to hypha-forming cells and
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21 phenotypic switching (i.e. the white-to-opaque transition). Both mechanisms facilitate
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23 adaptation of *C. albicans* to the changing environmental conditions encountered during
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25 colonization of new niches within the host. The main virulence factors of *C. albicans*
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27 include secreted hydrolytic enzymes, mainly phospholipases and proteinases, and the
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29 cytolytic peptide toxin—candidalysin that enable evasion of antimicrobial host defenses
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31 and contribute to pathogenesis by disruption of host cell membranes or degradation of
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33 host immune proteins or peptides (Naglik, Challacombe, Hube, 2003; Moyes *et al.*, 2016).
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41 One of the key features of candidiasis development is the adhesion of *C. albicans* to
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43 human epithelial or endothelial cells, during which both morphotypes can engage several
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45 adhesins and other surface-located proteins. Moreover, the adhesive properties of fungal
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47 cells facilitate the formation of biofilms, which promote persistent host colonization and
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49 increased resistance to host defenses or antimycotic treatments (Tsui, Kong, Jabra-Rizk,
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51 2016). Among the adhesins identified on the *C. albicans* cell surface, members of the
52
53 agglutinin-like sequence (Als) protein family (Als1-9) play an essential role in establishing *C.*
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55 *albicans*-host interactions (Hoyer & Cota, 2016). These proteins are
56
57 glycosylphosphatidylinositol (GPI)-linked to β -1,6-glucans of the fungal cell wall (Gaur &
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3 Klotz, 1997; Hoyer, 2001; Sheppard *et al.*, 2004); some are preferentially expressed by germ
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5 tubes and hyphae (Als1-4), and others by yeast-like cells (Als5-7, Als9) (Modrzewska &
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7 Kurnatowski, 2015). Proteins Als2, Als3, and Als4 have been identified as facilitating *C.*
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9 *albicans* adhesion to host cells and, for Als3, E-cadherin on epithelial cells or N-cadherin on
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11 endothelial cells were shown to be binding targets (Phan *et al.*, 2007). Host extracellular
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13 matrix (ECM) proteins, including fibronectin, laminin, collagen, gp96, EGFR and HER2, have
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15 also been identified as Als binding partners. Additionally, specific proteins of the blood
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17 coagulation cascade such as kininogen, plasminogen and fibrinogen were found to bind Als3
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19 (Gaur, Smith, Klotz, 2002; Gaur and Klotz, 2004; Sheppard *et al.*, 2004; Phan *et al.*, 2007;
20
21 Almeida *et al.*, 2008; Silverman, Nobbs, Vickerman, Barbour, Jenkinson, 2010; Karkowska-
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23 Kuleta *et al.*, 2011; Liu, Mittal, Solis, Prasadaraao, Filler, 2011; Zhu *et al.*, 2012; Seweryn *et*
24
25 *al.*, 2015).

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33 Alongside dedicated adhesins such as the Als family, the *C. albicans* cell surface is
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35 equipped with so-called “moonlighting” proteins. These are abundantly expressed at the
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37 cell surface but are not secreted via the classical secretory pathways. These factors include
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39 chaperones, stress protectants, and enzymes involved in intracellular processes such as
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41 glycolysis and gluconeogenesis, the citric acid cycle, the pentose phosphate pathway, and
42
43 the metabolism of DNA and proteins (Karkowska-Kuleta & Kozik, 2014; Jeffery, 2018;
44
45 Karkowska-Kuleta, Satala, Bochenska, Rapala-Kozik & Kozik, 2019). However, when
46
47 associated with the cell surface, these moonlighting proteins seem to perform alternative
48
49 functions. Several bacteria and fungi have been found to use moonlighting proteins as
50
51 receptors for host proteins, mediating physical attachment to the host cell surface or
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53 facilitating invasion, virulence, biofilm development or disseminated infection (Satala,
54
55 Karkowska-Kuleta, Zelazna, Rapala-Kozik, Kozik, 2020a).

One of the most crucial moonlighting proteins of *C. albicans* is enolase, an evolutionarily conserved enzyme of the glycolysis and gluconeogenesis pathways. Its extracellular function is important for infection propagation and seems to be universal amongst different fungal species, bacteria, and parasites (Jeffery, Bahnson, Chien, Ringe, Petsko, 2000; Ehinger, Schubert, Bergmann, Hammerschmidt, Heinz, 2004; Liu *et al.*, 2012; Chen, Zabad, Liu, Wang, Jeffery C, 2018; Karkowska-Kuleta *et al.*, 2018). These proteins present a high degree of sequence homology between enolases of evolutionarily distant organisms, including mammals (Hannaert *et al.*, 2003; Macías-Sánchez *et al.*, 2015).

Enolase also displays multiple functions within the cell. In the cytoplasm of mammalian, fungal and bacterial cells, enolase functions as a chaperone, and was previously proposed to be involved in the survival and proliferation of *Streptococcus mutans* at low pH (Wilkins, Homer, Beighton, 2002), *Escherichia coli* under oxidative stress (Tamarit, Cabiscol, Ros, 1998), and various yeasts under heat shock (Iida & Yahara, 1985). Previous studies also indicated an association between enolase and the mitochondrial membrane, and it has been ascribed as a RNA chaperone due to RNA binding activity (Brandina *et al.*, 2006; Entelis *et al.*, 2006). Enolase was also recognized as a constituent and regulator of vacuoles, especially in *Plasmodium falciparum*, where different Eno variants were involved in parasite survival in a hostile environment via the complex neutralization of free heme (Shevade, Jindal, Dutta, Jarori, 2013). In the yeast *S. cerevisiae*, both Eno1 and Eno2 proteins stimulate the fusion of vacuoles and probably perform a transporter function (Decker & Wickner, 2006; Wiederhold *et al.*, 2009).

Eno1 exposed at the *C. albicans* cell surface was involved in the binding of human plasminogen (Crowe *et al.*, 2003). It has also been found to interact with plasma contact factors: kininogen, factor XII and prekallikrein, leading to host-independent production of

kinins—the vasoactive peptides conducive to the spread of yeast infection (Seweryn *et al.*, 2015; Funk *et al.*, 2016). Several studies have implicated enolase as protein mediating microbial adhesion to host extracellular matrix proteins such as fibronectin, laminin and vitronectin (Carneiro, Postol, Nomizo, Reis, Brentani, 2004; Esgleas *et al.*, 2008; Castaldo *et al.*, 2009; Donofrio *et al.*, 2009; Satala *et al.*, 2020b) and promoting a fast humoral response in patients with systemic candidiasis (Strockbine, Largen, Zweibel, Buckley, 1984). Enolase was also proposed to be an important allergen in lung diseases (Ito, Ishiguro, Kanbe, Tanaka, Torii, 1995). Moreover, being commonly identified in the blood of infected patients, *C. albicans* enolase is used as a marker of invasive candidiasis (Walsh *et al.*, 1991). Unexpectedly, enolase localized on the *C. albicans* cell surface also shows transglutaminase activity, contributing to cell morphological changes and cell wall remodeling, but its exact role in the pathogenicity of these fungi is not fully understood (Reyna-Beltrán *et al.*, 2018).

C. albicans enolase has been detected on the surface of both blastospores and hypha, where its association with cell wall glucans was proposed (Angiolella *et al.*, 1996). Although little is known about the transport of enolase to the fungal cell surface, studies of post-translational modifications that can occur during this process for human and bacterial enolases have been reported (Boël *et al.*, 2004; Daubenspeck, Liu, Dybvig, 2016; Zakrzewicz *et al.*, 2018). It has also been shown that enolase can be secreted outside the *C. albicans* cells as a cargo in extracellular vesicles (Gil-Bona *et al.*, 2015; Vargas *et al.*, 2015; Wolf, Espadas, Luque-Garcia, Reynolds, Casadevall, 2015). Silva and coworkers (2014) documented the presence of enolase within biofilm structures and demonstrated that this did not arise from cell lysis and subsequent protein readsorption to the cell surface. Their analyses also showed that blocking of cell surface enolase by protein-specific antibodies inhibited the adherence of *C. albicans* cells to mice GI tissue (Silva *et al.*, 2014).

Given the limited understanding of the regulation and role of enolase at the fungal cell surface this study aimed to analyze enolase exposure at the *C. albicans* cell surface under different growth conditions and the potential for readsorption of cytosolic enolase. The role of external enolase in mediating the interactions of fungal cells with host plasma proteins involved in the blood coagulation/fibrinolysis cascades, human plasminogen (HPG) and human high molecular mass kininogen (HK), was also experimentally addressed.

2. Results

2.1. Enolase is exposed on the *C. albicans* yeast-like cell surface during the stationary phase of growth

The origin of the enolase that has been repeatedly identified at the surface of *C. albicans* yeast-like cells, hyphae, and biofilm-forming cells (Pancholi, 2001; Silva *et al.*, 2014) still remains controversial. Hence, in our present study, we conducted a stepwise analysis of the surface appearance of enolase, using *C. albicans* strain ATCC10231 grown in YPD medium at 30°C. Under these growth conditions, the cells were in a yeast-like form and did not form hyphae. The progress in cell growth was determined by optical density measurements carried out concurrently for 19 hours with a determination of cell viability using the XTT colorimetric assay for cellular metabolic activity (Figure 1). The appearance of enolase at the *C. albicans* cell surface was detected with a specific antibody and reached its highest level after 19 hours of growth under the conditions studied, in which the cells had reached the stationary growth phase. Monitoring of enolase levels over the longer time scale showed no significant changes in the amount of this protein, which remained almost constant on the surface of *C. albicans* cells (Figure S1 in Supplementary materials).

2.2. The fungal cell environment influences the surface localization of enolase

2.2.1. Enolase display at the candidal cell surface depending on the growth conditions

As the environment around *C. albicans* cells may affect the yeast metabolism and physiology, we evaluated the possible modulation of enolase display at the fungal cell surface at variable conditions of cell growth. For the detection of *C. albicans* surface-displayed enolase after culture in different media in shaking flask cultures, where cells are more likely to grow in a planktonic form, cell surface shaving with trypsin and liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) were used (Figure 2A; mass spectrometric details for protein identification are included in Supplementary Table 1). This method of identifying proteins and estimating their relative abundance at the cell surface from the calculation of the normalized spectral abundance factors (NSAFs) (Zybailov, Coleman, Florens and Washburn 2005; Zybailov, Florens, and Washburn 2007) has been successfully used previously for *C. albicans* cells (Gil-Bona *et al.*, 2015; Marín *et al.*, 2015). Relative enolase display at the surface of the fungal cells grown in different media was compared with cells propagated aerobically in YPD medium, which is commonly and routinely used for yeast culture. Some of the tested growth media simulated roughly the conditions that prevail in different infectious niches such as artificial saliva (AS) and artificial urine (AU), thus providing some approximation of the conditions under which infections occur in the oral cavity or urinary tract, respectively. In addition, enolase exposure during growth under anaerobic conditions in YPD medium was also tested (AN).

Even considering that the detection of enolase was semi-quantitative, this protein was identified at comparable levels on the surfaces of *C. albicans* cells cultured under very different conditions, suggesting that its surface location is a general phenomenon. The

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highest level of changes in the surface presence of enolase was noted for medium SDL, containing casein. However, as that was a relative measurement, it is possible to some degree that it may reflect not only the accumulation of enolase at the cell surface but, additionally, the loss of other surface proteins. A complete list of proteins for each culture condition is presented in Supplementary Table 1.

C. albicans cells growing at the place of infection in mono-species or multi-species consortia prefer to form a biofilm regulated by quorum sensing molecules (QSM). Using immunodetection and flow cytometry, we tested whether the process of enolase display at the surface of *C. albicans* cells could be affected by the fungal or bacterial quorum-sensing molecules, farnesol (F) and farnesoic acid (FA), the autoinducer-2 (AI-2), or the major lipopolysaccharide (LPS) component of the outer membrane of Gram-negative bacteria that fungi may come into contact with during the creation of a mixed-species biofilm (Bandara, Yau, Watt, Jin, Samaranayake, 2009). As the involvement of enolase in response to oxidative stress was indicated previously (Serrano-Fujarte, López-Romero, Cuéllar-Cruz, 2016), the effect of exogenous H₂O₂ on enolase presence at the surface of *C. albicans* cells was also tested (Figure 2B). Enolase was found under all of these conditions at a high but comparable level to the control cultures of *C. albicans* blastospores grown in YPD medium for 16 hours.

2.2.2. Morphology of *C. albicans* influences the enolase display on the fungal cell surface

The presence of enolase at the cell surface was next analyzed during the growth of the different morphological forms of *C. albicans* cells i.e. yeast-like cells (blastospores) vs. filamentous forms.

Since a definitive change in the morphological form usually results from a significant change in growth conditions, we initially estimated the direction of these changes using *C. albicans* cells growing in the Saboraud medium for 16 h, but under different thermal conditions (30°C versus 37°C) that trigger filamentation. The enolase cell surface display was determined using anti-enolase primary antibodies and secondary antibodies conjugated with HRP and TMB as a substrate for HRP. At a temperature of 37°C we observed an increase of enolase presence on the cell surface, correlating with the initiated filamentation (Figure S2 in the Supplementary materials). Another approximation was made with the cells growing in YPD medium enriched with selected plasma proteins at a concentration corresponding to their presence in plasma. As protein content drives *C. albicans* cell filamentation, we observed an accompanying increase in enolase display on the hyphae surface (Figure S3 in the Supplementary materials). In further experiments we used unicellular yeast cells, obtained after growth in YPD medium as described above, whereas the hyphal forms of *C. albicans* cells were produced in microplate wells or on coverslips coated with poly-L-lysine, in RPMI 1640 medium for 3 hours at 37°C. The presence of enolase at the cell surface was then tested microscopically (Figure 3A,B), with specific fluorescently labeled antibodies, or spectroscopically (Figure 3C), using the antibody conjugated with HRP. These two methods have been chosen to account for differences when comparing the surface area of the different morphological forms of fungal cells. The fixation of the cells was omitted to avoid non-specific binding. The fluorescence intensity measurements were calculated per surface area of each cell, determined according to the modified method presented by Urban and co-workers (Hosseinzadeh & Urban, 2013) using the Olympus Cell Sense software (Figure 3B). Similar calculations of cell surface area were made also for the second experimental system where enolase detection was performed with the HRP/TMB system. Taking into

account the calculations carried out for the surface area of both types of cells, in this experiment a 10-fold excess of blastospores was used in relation to the number of cells forming hyphae. The detailed calculations were presented in the Supplementary materials in Figure S4. The amount of surface-located enolase was reported as the absorption of converted TMB substrate (Figure 3C).

In both approaches, greater enolase abundance was found at the surface of hyphal forms, indicating a preference for these morphological forms. This might also be indirectly related to the generally greater abundance of intracellular enolase in hyphal forms than in blastospores—2% vs. 0.7% of the total protein content, respectively, detected by Sundstrom and Aliaga (1994).

2.3. Excreted enolase is re-adsorbed on the surface of the filamentous form of *C. albicans* cells

Since enolase is found at the surface of *C. albicans* cells, although its secretion pathways can be variable and have not yet been identified in detail, such as a possible origin from surrounding dead cells or as a cargo of extracellular vesicles, the possibility of the readsorption of enolase from the outside of the cell to its surface was tested in our current experiments (Figure 4). For this analysis, enolase purified from the cytosol of *C. albicans* cells was employed as it more closely reflects the biological and functional state of this protein. It was previously shown that cytosolic enolase can adsorb to *C. albicans* cell walls that were isolated after breaking the cells with glass beads (Eroles, Sentandreu, Elorza, Sentandreu, 1997). Nevertheless, in our present study we used whole, intact fungal cells,

and found that the association of exogenous enolase was undeniably apparent for both morphological forms of *C. albicans*. We used fluorescein-labeled enolase for microscopic observations or biotin-labeled enolase for the detection with the SA-HRP/TMB system. We further observed an increase in the amount of enolase deposited at the cell surface upon exposure to higher concentrations of added protein. These findings also confirmed that more enolase can be readsorbed to the surface of the hyphal forms of fungal cells than to blastospores. Importantly, this could be related to the change in the proteome of the surface of *C. albicans*, which has been frequently identified in connection with changes in the morphological forms of fungal cells (Martínez-Gomariz *et al.*, 2009; Heilmann *et al.*, 2011). This raises a crucial question as to whether particular proteins located at the surface of *C. albicans* cells are receptors or binding proteins for secreted enolase. We thus next searched for potential proteinous sites for enolase deposition at the fungal cell surface.

2.4. The *C. albicans* surface adhesin Als3 is involved in the binding of enolase

Two complementary methods were used for the isolation and identification of putative enolase-binding proteins from mixtures of proteins extracted from the surface of *C. albicans* cells grown in the form of germ tubes and hyphae. One method involved the use of affinity chromatography with agarose-coupled fungal enolase, whilst the second was based on chemical cross-linking with a heterobifunctional, photoactivated reagent and biotin-labeled *C. albicans* enolase. Only the protein bands that were not present in the control sample were excised from the electrophoretic gel and subjected to in-gel tryptic digestion. The obtained results thus indicate the most frequently identified putative interaction partners for *C. albicans* enolase (Table 1; detailed parameters from mass spectrometry identification are included in Supplementary Table 2). Among the typical cell wall proteins covalently

bound to the fungal cell surface, primarily Als3 was indicated as highly capable of binding to enolase, as it was identified four times, both after using chromatography and cross-linking. Other proteins, which were identified similarly often or with a slightly lower frequency, were, like enolase, atypical cell wall proteins and their presence in the samples may have been related to similar mechanisms of deposition at the cell surface, an affinity for enolase, or a mutual affinity related to the formation of known complexes of several glycolytic enzymes (Kurganov, Sugrobova, Mil'man, 1985; Araiza-Olivera *et al.*, 2013; Menard, Maughan, Vigoreaux, 2014).

For testing the possible contribution of Als3 to enolase localization at the surface of fungal cells, *S. cerevisiae* cells overexpressing Als3 protein were used, and the result was compared with the enolase binding properties of other GPI anchored *C. albicans* proteins (Eap1 and Hwp1) considered as references in the same model (Figure 5). These *C. albicans* adhesins were expressed at the surface of the *S. cerevisiae* surrogate as fusion proteins with a hemagglutinin (HA) tag near the C terminus, followed by the Ser/Thr-rich region of Epa1 from *C. glabrata* and the C-terminal domain of the *S. cerevisiae* protein Cwp2, introduced for efficient anchoring to the cell wall (Nobbs, Vickerman, Jenkinson, 2010). By using anti-HA antibodies, we verified that these proteins had a comparable surface expression level between individual experiments. A control strain expressing the *S. cerevisiae* Cwp1 protein was used to test the possible effect of an additional fragment of the *C. glabrata* protein on adhesion and protein binding. Whilst maintaining a similar level of expression of *C. albicans* adhesive proteins at the surface of *S. cerevisiae*, the highest enolase binding capacity was demonstrated for the Als3 protein, confirming our earlier observations (Table 1), whereas the other *C. albicans* proteins tested—Eap1 and Hwp1—could make a contribution, although significantly lower, to interactions with cytoplasmic enolase at the fungal cell

surface. The low signal obtained for enolase binding to Cwp1 excluded the participation of the other supporter fragments of the construct in these interactions, as well as any significant contribution of the other components of the surrogate *S. cerevisiae* cell surface.

2.5. Characteristics of the enolase-Als3 interaction: binding kinetics and involved adhesin molecule fragments

To verify that Als3 interacts with enolase, as indicated by the affinity chromatography, cross-linking and experiments using *S. cerevisiae* cells exposing selected candidal adhesins, we purified the Als3 protein from surface of *C. albicans* cells using a previously described method (Seweryn *et al.*, 2015). The surface plasmon resonance (SPR) measurements were employed in which the Als3 protein was deposited on the surface of a CM3 chip in accordance with the manufacturer's protocol to better reflect its surface-bound state and to simplify the model of interaction. As a reference, a second cell on the CM3 chip was activated with the EDC/NHS system and blocked with ethanolamine. Based on the resonance curves obtained during contact of the immobilized Als3 protein with a number of enolase dilutions, the dissociation constant of the formed complex was estimated at 36.7 nM using homologous plots of association and dissociation curves, representing the Langmuir model of interaction (Figure 6). A high binding affinity between these two proteins was thus confirmed.

To address the question of which parts of the Als3 molecule were principally involved in the interaction with enolase, we used *S. cerevisiae* cells expressing the full sequence or deletion mutants of Als3 (Figure 7A). These mutants were used previously to map the regions of Als3 responsible for adhesion to artificial surfaces, and thus essential for biofilm formation, and that participate in the interaction with ECM proteins (Silverman *et al.*, 2010;

Bamford, Nobbs, Barbour, Lamont, Jenkinson, 2015). In the method used in our present study, *S. cerevisiae* cell suspensions were allowed to equilibrate with solutions containing biotinylated enolase at increasing concentrations over the same range as in the experiments described above. The surface exposure of all Als3 variants on *S. cerevisiae* cells was determined in each experiment using antibodies that recognize a C-terminal HA-tag, as described previously (Nobbs *et al.*, 2010). A comparison of enolase binding to these mutants, presented in Figure 7B, was performed for the same enolase concentration (250 nM), ensuring the saturation of the binding sites. The binding to the full Als3 sequence on the *S. cerevisiae* cells was assigned a value of 100%, whereas that to the yeast cells expressing plasmid alone was taken as 0%. We found that the Ig-like N-terminal region (aa166-225; aa218-285; aa270-305; 277-286) and the central repeat domain (aa434–830) were essential regions of Als3 for the interaction with enolase as the corresponding mutants showed only about 20% binding. In contrast, the elimination of the sequence across the amyloid-forming region of Als3, aa325-331, which is believed to induce adhesin clustering (Beaussart *et al.*, 2012), did not reduce enolase binding.

2.6. The surface-located enolase of *C. albicans* is involved in the regulation of fungal Als3 adhesin interactions with host plasma proteins

The enolase binding to Als3 raised questions regarding the mechanism of its interactions with host proteins on the *Candida* cell surface. The most possible is an Als3-mediated bridging of enolase-host protein complexes on the fungal cells. To test this, we employed *S. cerevisiae* cells expressing surface *C. albicans* Als3 protein deprived of its amyloid sequence, as this variant has the same enolase binding properties as full-length Als3. The use of this deletion mutant overcomes the problem of yeast cell aggregation

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3 during FACS analysis. The cells were allowed to interact for 90 minutes with combinations
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5 of the fluorescein-stained host proteins HK or HPG, and Alexa Fluor 647-stained enolase, at
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7 different concentration ranges. After cell washing, the stained cells were analyzed for the
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9 presence of both types of proteins at their surface to examine for mutual protein
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11 interactions (Figure 8).
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15 At the low concentration of enolase (below 100 nM) in the cell surroundings, an
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17 increased deposition of enolase on the cell surface was observed. And it was not affected
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19 by the presence of any host proteins (Figure 8A, B). This concentration range of enolase
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21 corresponds to its relative binding constant (36.4 nM), estimated as the concentration of
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23 enolase that will achieve a half-saturation of binding sites on the *S. cerevisiae* cells (3×10^7
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25 cells) harboring surface Als3 molecules (Figure 5). It was consistent also with the value
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27 determined in our SPR experiment using immobilized Als3 protein (36.7 nM, Figure 6).
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29 However, with a further increase in the enolase concentration (above 100 nM), we
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31 observed a lowering of its surface binding in the presence of both host proteins. Moreover,
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33 the enolase binding to the Als-3 exposing cells was more sensitive to kininogen than to
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35 plasminogen presence, at the same concentration range of host proteins. These observed
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37 effects may have been the result of the possible formation of the soluble complexes
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39 between enolase and a specific host protein that lowered the level of enolase available for
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41 Als3 saturation at the yeast cell surface.
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50 Another explanation for these observed results assumes that enolase and host proteins
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52 can compete for a binding site on the Als3 molecule. A possible interaction between Als3
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54 and HK was identified in our previous study (Seweryn *et al.*, 2015), but the participation of
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56 Als3 in HPG binding by *C. albicans* has never been proposed. Hence, we verified an HPG
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58 interaction with Als3 and compared its binding affinity with that determined for HK using
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3 biotinylated plasma proteins contacting pBC-Als3 yeast cells (Figure 8C). This comparative
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5 analysis revealed that both proteins could bind to surface-exposed Als3 but with different
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7 binding strengths as a greater affinity of Als3 was detected for HK compared with HPG. To
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9 map the Als3 regions involved in the interaction with both host proteins, we again used cells
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11 expressing various deletion mutants of Als3 on their surface (Figure 8D). We concluded that
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13 similar regions of the Als3 molecule are essential for the binding to both host proteins.
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15 However, the interaction with HK was more sensitive to the elimination of any part of the
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17 Als3 molecule than that with HPG, suggesting tighter Als3 binding to HK than to HPG. This
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19 was also reflected by the above-demonstrated saturation of binding to *S. cerevisiae* pBC-
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21 Als3.
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28 We finally evaluated the host protein interactions with the yeast cells in the presence
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30 of an increased concentration of enolase. The results, presented in Figures 8E and 8F,
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32 confirmed the possible competition of host proteins and enolase for the same binding site.
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34 Moreover, the competitive potential of enolase towards HPG was higher compared with
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36 HK. These findings shed new light on the possible role of enolase at the *C. albicans* cell
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38 surface and highlight for the first time that the regulatory properties of enolase include the
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40 surface adsorption of host proteins essential for fungal infection.
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46 3. Discussion

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48 The multiplicity and diversity of *a priori* non-characteristic functions mediated at the
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50 surface of various cells by “moonlighting” proteins have led to the search for conditions that
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52 determine their unexpected location and interactions, i.e. that underlie their new roles.
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54 Enolase is one of the most common of these proteins and is found at the surfaces of almost
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56 all types of cells i.e. from bacteria (Pancholi & Fischetti, 1998; Derbise, Song, Parikh,
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3 Fischetti, Pancholi, 2004), fungi (Eroles *et al.*, 1997) and parasites (Bhowmick, Kumar,
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5 Sharma, Coppens, Jarori, 2009) to human immune (Wygrecka *et al.*, 2009), cancer
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7 (Didiasova, Wujak, Wygrecka, Zakrzewicz, 2014; Didiasova *et al.*, 2015) and neuronal
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9 (Haque, Polcyn, Matzelle, Banik, 2018) cells.
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13 The location and role of the multifunctional enolase protein at the fungal cell surface
14 remain enigmatic. The principal question thus concerns the conditions that favor enolase
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16 deposition on the yeast cells. As the scientific literature currently provides incomplete
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18 information on this topic, we first determined the cell growth phase and preferred medium
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20 for *C. albicans* ATCC10231 strain propagation that would result in the maximal accumulation
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22 of enolase on the cell surface. We documented that the long-lasting stationary phase of the
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24 cell growth promoted enolase deposition on the yeast-like *C. albicans* cells. For further
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26 comparison, we used several different growth media that simulated the conditions found
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28 in natural niches settled by *C. albicans* and did not find significant differences in the enolase
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30 surface content compared with cells growing in YPD media, although some variation in this
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32 regard was observed for non-*albicans Candida* species (Karkowska-Kuleta *et al.*, 2019).
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40 Silva and co-workers (Silva *et al.*, 2014) had found in their prior study that enolase was
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42 present in the matrix of mono-species fungal biofilms. However, *C. albicans* also forms
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44 mixed biofilms with bacteria, such as anaerobic *P. gingivalis*, that is well protected in aerobic
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46 conditions by fungal cells (Bartnicka *et al.*, 2019). We noted increased surface display of
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48 enolase under anoxia and in aerobic cell growth in the presence of bacterial cells compared
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50 with a mono-species fungal biofilm (Bartnicka *et al.*, 2019). There are a plethora of factors
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52 that might be responsible for this phenomenon. First of all, there are the quorum sensing
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54 molecules (QSM) produced by the action of both types of pathogens to influence their own
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56 or their partner's propagation, including fungal farnesol and farnesoic acid, as well as
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3 bacterial autoinducer-2. There were no significant changes however in the surface enolase
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5 levels on the cells treated with both types of QSM at the concentration range identified
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7 under mixed biofilm conditions. Surprisingly also, we found in our present analyses that
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9 bacterial LPS did not influence the enolase level on the fungal cell surface. However, in the
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11 case of human cells, it was shown previously that LPS can trigger the translocation of host
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13 enolase to the extracellular space due to fluctuations in the intracellular calcium level
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15 (Didiasova *et al.*, 2015) or post-translational modifications (Zakrzewicz *et al.*, 2018). Another
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17 stimulating factor that may potentially influence the surface deposition of fungal enolase is
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19 the proteolytic activity of bacteria. Indeed, a previous comparison of mixed biofilms formed
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21 by fungi with the wild type *P. gingivalis* W83 strain or its mutant deprived of the leading
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23 group of cysteine proteases, the gingipains, revealed a two-fold lower level of surface fungal
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25 enolase during contact with the mutant strain, but still at a higher level than in mono-
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27 species fungal biofilms formed under the same conditions (Bartnicka *et al.*, 2019).
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35 The increasing bacterial proteolytic activities resulted in a gradual loss of fungal cell
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37 integrity. This may point to enolase deposition after its outflow from damaged cells. Notably
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39 however, Silva and co-workers (Silva *et al.*, 2014) demonstrated that the enolase from lysed
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41 *C. albicans* cells of strain 5373 was not stable outside the cells. On the other hand, the
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43 isolation of intact protein from the biofilm structure formed by the cells of strain 3232
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45 suggested that the quick binding of enolase to the outer fungal cell surface or biofilm matrix
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47 can protect the protein from further degradation.
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53 There has only been one publication to date (Angiolella *et al.*, 1996) showing that
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55 enolase is associated with glucans in the cell wall. However, to identify the surface attached
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57 proteins, the authors of that study used a β -1,3-glucanase (zymolyase) in the extraction
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59 method. This enzyme also contains mannanase activity that may partially deglycosylate
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3 some of the surface proteins that could play a receptor function for enolase (Beucher *et al.*,
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5 2009). Moreover, these authors did not analyze high molecular mass proteins or identify
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7 any complexes.
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10 Before looking for an enolase receptor at the *C. albicans* cell surface in our present
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12 analysis, we first tested the possibility that enolase rebinds to the cell surface, as its
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14 transport not only to the cell surface but also into the medium has been commonly
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16 observed (Sundstrom & Aliaga, 1994; Eroles *et al.*, 1997). As expected, our analysis revealed
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18 that the filamentous form of the *C. albicans* fungal cells bound a higher amount of enolase
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20 at the cell surface compared with the yeast-like cells.
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25 We searched for a possible receptor capable of enolase binding using two independent
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27 approaches, and we found that Als3—the GPI anchored glycoprotein—and some cellular
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29 enzymes were putative enolase binding proteins. Among them, glucose-6-phosphate
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31 isomerase, 6-phosphogluconate dehydrogenase, and phosphoglycerate mutase belong, like
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33 enolase, to the “moonlighting” protein group. Besides, it was documented that mouse
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35 muscle enolase binds with a high affinity to some glycolytic enzymes, like phosphoglycerate
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37 mutase or pyruvate kinase (Merkulova *et al.*, 1997), suggesting that these enzymes can be
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39 transferred or located in the form of complexes at the fungal cell surface, possibly stabilizing
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41 their structural integrity. Taking into account that these enzymes are rather responsible for
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43 translocation or stability of extracellular enolase, in the further analysis we focused on Als3
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45 protein as a putative receptor. In addition, among the typical cell wall proteins, chitinase 2
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47 was also identified (Dünkler, Walther, Specht, Wendland, 2005) but its contribution to
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49 enolase binding was by far the lowest.
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56 We verified the participation of Als3 in enolase attachment by testing enolase binding
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58 to *S. cerevisiae* cells that expressed different *C. albicans* surface adhesins, including Als3,
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Eap1, and Hwp1. These experiments revealed that only Als3 was able to tightly interact with enolase. We used the popular *S. cerevisiae* model cell system to overexpress the *C. albicans* *ALS3* gene instead of *C. albicans* deletion mutants, to avoid possible compensatory changes in the expression of other *ALS* genes, but still keeping in mind the possibility of altered glycosylation patterns from this exogenous expression (Cota & Hoyer, 2015).

The hypothesis that Als3 is the major Als receptor for enolase is supported by the findings that the gene encoding this protein has a higher transcriptional level in all types of *C. albicans* strains, independent of their growth milieu. Moreover, under conditions that promote hyphal growth, anti-Als3 immunostaining pointed to peak Als3 expression in cells adopting this morphology (Coleman *et al.*, 2009), with different location preferences for other members of this protein family. The Als3 protein is linked to β -1,6-glucan (Kapteyn *et al.*, 2000), which explains the previously observed release of enolase from the fungal cell surface after enzymatic treatment with zymolyase.

Als3 possesses an extensive list of divergent, structurally unrelated binding partners among host proteins as well as bacterial proteins such as SspB of *Streptococcus gordonii* (Silverman *et al.*, 2010) and InlJ or Rgp of *P. gingivalis* (Sztukowska *et al.*, 2018; Bartnicka *et al.*, 2019). The collective evidence to date suggests that the principal binding regions within the Als3 molecule are localized within its N-terminal domain (NT), which possesses broad peptide recognition properties (Salgado *et al.*, 2011) and was found to be important for interactions with host proteins such as the E- or N-cadherins (Phan *et al.*, 2007), collagen, fibrinogen or fibronectin, as well as the *S. gordonii* SspB protein (Bamford *et al.*, 2015). On the other hand, the amyloid-forming region (AFR), localized at the very end of the NT domain of Als3, and found to be critical for the *C. albicans* cell aggregations (Lin *et al.*, 2014), is not crucial for interactions with bacterial SspB and its deletion only partially affected the

biofilm formation by Als3-exposed *S. cerevisiae* cells on silicone surfaces coated with ECM proteins. The central repeat domain (CRD) of Als3 was found not to be required for bacterial adhesion, but its deletion affected the interaction with ECM proteins, indicating a possible role of this domain in the folding of the N-terminal region of Als3 or the exposition of additional adhesive areas in the Als3 molecule (Bamford *et al.*, 2015).

A similar strategy in identification of Als3 fragments important for the interaction with *C. albicans* enolase showed that this protein preferred to interact with the same regions of the Als3 molecule as ECM, without any binding to the AFR region and with stronger preference to the CRD fragment. Comparing all these results, it could be concluded that Als3 molecule interacts relatively easily with proteins of various characteristics, presenting broad substrate specificity. The immobilization of purified Als3 on the chip surface enabled the determination of its enolase binding properties, characterized by the dissociation constant of 36.7 nM. This is the first information about the possible mechanism of enolase display at the fungal cell surface, with involvement of typical fungal adhesins, and a possible explanation of enolase surface location.

The main role of enolase exposed at pathogen cell surfaces has been attributed to the binding of host plasminogen, which can facilitate pathogen migration through the host tissues and consequently the progression of infection (Irigoyen, Muñoz-Cánoves, Montero, Koziczak, Nagamine, 1999; Crowe *et al.*, 2003). Plasminogen in the host plasma is converted to plasmin, which further degrades fibrin but can also digest extracellular matrix proteins and activate collagenases. Plasminogen bound to fungal cells can also be activated to plasmin by its treatment with specific, externally added activators i.e. tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Irigoyen *et al.*, 1999; Jong *et al.*, 2003).

The possible role of enolase as “a bridging molecule” between the fungal adhesin Als3 and host plasminogen is an attractive hypothesis, but its actual functioning seems to be much more intricate. Using enolase and plasminogen labeled with different fluorescent dyes and yeast cells overexpressing *C. albicans* Als3, we here identified, for the first time, how such interactions can be executed. We first observed that an increased host protein concentration caused a decrease in enolase binding to Als3-exposed *S. cerevisiae* cells. On the other hand, the binding of host proteins in this system was also observed, suggesting a potential interaction between Als3 and host proteins. For the results identified for plasminogen, we used kininogen (HK) as a sort of reference host protein, the binding of which on *Candida* spp. cells was well documented (Karkowska-Kuleta, Kozik, Rapala-Kozik, 2010; Karkowska-Kuleta *et al.*, 2011). HK binding by fungal enolase, as well as by Als3, has been previously observed and characterized by dissociation constants (K_D) in the same range for both fungal proteins (ca. 200 nM). These results confirmed the possible competition of HK and enolase for the binding site on Als3 molecule. Similar results were obtained for plasminogen in our present analysis, indicating that Als3 can also function as a receptor for this host protein. Plasminogen binding to Als3 was previously reported only for *C. parapsilosis* homologs of Als adhesin, with the reported kinetic parameters in a comparable range (Karkowska-Kuleta *et al.*, 2017).

It is noteworthy that the identification of Als3 in cell wall protein (CWP) extracts is quite difficult and depends on the extraction method used, electrophoresis conditions, as well as the protein staining procedures. This is likely to explain why this protein has rarely been identified in prior mass spectrometry analysis of *Candida* cell wall proteins that may participate in host protein binding. To further verify this observed interaction, we mapped the regions of Als3 involved in the interaction with HPG and HK and found that the same

domains of this protein required for enolase binding were also essential for interactions with these host proteins. This supports the possibility of a competition between enolase and host proteins for binding to Als3. Moreover, our current observation that HK has a higher binding affinity for Als3 than HPG in the *S. cerevisiae* model was consistent with our finding of the stronger competition between enolase and HK compared with the enolase-HPG pair in the binding to Als3.

The involvement of the surface Als3 protein and enolase, both at the fungal cell surface, as well as those found in solution, may play an important role in the subtle modulation of discussed processes by *C. albicans* and thereby tip the balance in favor of the pathogen. Hence, the interactions of these fungal and human proteins and their further impact on the development of infection is multifaceted and requires further detailed research.

4. Experimental procedures

4.1. Fungal strains and growth conditions

Candida albicans strain ATCC® 10231™ (3147) was purchased from American Type Culture Collection (Manassas, VA). *Saccharomyces cerevisiae* strains used in this study are listed in Table 2. *C. albicans* cells were routinely cultured with shaking (170 rpm) in a liquid YPD medium (1% yeast extract, 2% soybean peptone and 2% glucose; Sigma, St. Louis, MO) at 30°C for 16 hours. *S. cerevisiae* strains expressing *C. albicans* adhesins were cultured without uracil at 30°C for 48 hours in a liquid complete synthetic medium (CSM) (Formedium, Norfolk, United Kingdom) supplemented with 0.67% yeast nitrogen base and 2% glucose (Sigma), whereas for cultures of the parent strain *S. cerevisiae* BY4742, a supplementation with 25 µg/mL uracil was applied under the same conditions (Beaussart *et al.*, 2012; Bamford *et al.*, 2015). Cell numbers for both species were determined by optical

density measurements at 600 nm after harvesting the cells by centrifugation (3000 *g*, 5 minutes) and washing three times with 1 mL of PBS buffer, pH 7.4. The appropriate growth medium dedicated to particular yeast species with the addition of 1.5% agar was used to store the fungal cultures.

4.2. Determination of *C. albicans* growth curve and cell viability

Samples of *C. albicans* yeast cells (10^7 cells/mL) were placed into MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) in 200 μ L of YPD medium per well and then incubated under cover at 30°C with shaking in a Synergy™ H1 microplate reader (BioTek Instruments, Winooski, VT) for 18 hours. The OD at 600 nm was measured every hour. Additionally, to test the cell viability, aliquots containing the same number of cells were plated into the wells at 2-hour intervals and incubated in YPD medium at 30°C. After 19 hours of incubation, the cells were washed and incubated with tetrazolium dye (XTT) (Thermo Fisher Scientific, Waltham, MA) for 2 hours at 30°C, and the absorbance was measured at 450 nm.

4.3. Immunodetection of surface-located *C. albicans* enolase

C. albicans yeast cells grown were transferred into Eppendorf tubes and washed twice with 1 mL of PBS. Then 100 μ L aliquots of cell suspensions containing 10^7 of yeast-like cells were placed into new tubes. The hyphal forms of *C. albicans* were obtained after a further incubation of the fungal cells (10^6) for 3 hours in 100 μ L of RPMI 1640 medium at 37°C in MaxiSorp microplates (Nunc). Prior to the addition of the antibodies, to avoid their non-specific binding, the surface of the fungal cells, both yeast-like and hyphal forms, as well as the unoccupied microplate well surface were blocked by incubation with 300 μ L of 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Moreover, the planktonic cells were

transferred after the binding of antibodies to new Eppendorf tubes prior addition of the substrate for peroxidase. For detection of enolase at the surface of yeast-like cells growing in 100 μ L of YPD medium in the wells of the microplate, 10^7 cells/mL were fixed with 100 μ L of 3.7% paraformaldehyde for 30 minutes at appropriate time intervals, to stop further cell growth. At each timepoint the yeast cells in the tubes were centrifuged (5 minutes, 3000 g) and washed three times with 100 μ L of PBS, and the cells on the microplate were washed three times with 200 μ L of PBS. The detection of surface-displayed enolase for both morphological forms was performed with the addition of 50 μ L of mouse monoclonal anti-enolase antibodies (1 μ g/mL) in PBS (Invitrogen, Carlsbad, CA) for 1 hour at 37°C, followed by 50 μ L of goat anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP) (1 μ g/mL, Abcam, Cambridge, UK) for 1 hour at 37°C. The enzymatic reaction was developed with 50 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) as the HRP substrate for 3 minutes and stopped with the addition of 50 μ L 2 M HCl. The supernatant was then transferred into a new microplate and the absorbance at 450 nm was measured using a Power Wave X Select microplate reader (BioTek Instruments).

An analogous procedure was used for microscopic observations; however, to obtain *C. albicans* hyphal forms, blastospores from a 16-hours culture were transferred to the wells of a glass bottom 96-well plate (CellVis, Mountain View, CA, USA) coated with poly-L-lysine (10^6 cells per well) and incubated in RPMI 1640 for 3 hours at 37°C. The next steps were common to both morphological forms. After washing with PBS, cells were incubated with 1% BSA, then with 50 μ L of mouse monoclonal anti-enolase antibodies (1 μ g/mL, Invitrogen) in PBS at 37°C for 1 hour and, subsequently, with Alexa Fluor 488 fluorescently labeled donkey anti-mouse secondary antibodies (1 μ g/mL, Abcam, Cambridge, UK) in PBS for 1 hour. The unbound antibodies were washed away with PBS. Blastospores were transferred

from Eppendorf tubes into the wells of a 96-well plate (CellVis) and centrifuged. Imaging was performed using an Olympus IX73 microscope (Olympus, Tokyo, Japan) equipped with a Hamamatsu Orca Spark camera (Hamamatsu, Hamamatsu City, Japan) and a UPLXAPO60XO lens (Olympus). Image analysis was performed using CellSense software (Olympus). To avoid changes in fluorescence intensity due to image acquisition parameters, all images were captured with the same integration times, excitation light intensity, and camera sensitivity. The fluorescence intensity for individual cells (blastospores and filamentous forms) was analyzed (Allikalt, Laasfeld, Ilisson, Kopanchuk, Rinken, 2020). The obtained mean fluorescence intensity (the total fluorescence intensity of the selected area divided by the cell area) determined by a method proposed by Hosseinzadeh and Urban (Hosseinzadeh & Urban, 2013) depends only on the amount of bound fluorophore. The cell borders were annotated manually as one pixel wide lines, in pictures taken in a bright field (Supplementary file – Figure S4). Only the hyphae or their fragments directly in the plane of focus were analyzed.

4.4. Identification of *C. albicans* surface enolase by tryptic shaving

After the growth of *C. albicans* cells in YPD medium at 30°C for 16 hours, aliquots containing 5×10^8 cells were transferred to 20 mL of different growth media and further cultured aerobically for 16 hours or anaerobically for 72 hours at 37 °C (30°C only for the control culture in YPD) on an orbital rotary shaker MaxQ 6000 (170 rpm) (Thermo Fisher Scientific). Anoxic conditions were obtained using a GENbox jar with a GENboxanaer generator (bioMérieux SA, Marcy l’Etoile, France). The following media were used for subsequent cultures: (i) AS – artificial saliva comprising 2.5 g/L mucin from a porcine stomach, type III, 10.0 g/L animal peptone, 5.0 g/L trypticase peptone, 5.0 g/L yeast extract,

5 mg/L hemin, 1 mg/L menadione, 60 mg/L urea (Sigma), 174 mg/L arginine (BioShop) and 2.5 g/L KCl, pH 7.0; (ii) AU – artificial urine composed of 0.65 g/L CaCl₂, 0.65 g/L MgCl₂, 4.6 g/L NaCl, 2.3 g/L Na₂SO₄, 0.65 g/L Na₃C₃H₅O(CO₂)₃, 0.02 g/L Na₂C₂O₄, 2.8 g/L KH₂PO₄, 1.6 g/L KCl, 1.0 g/L NH₄Cl (Avantor Performance Materials Poland S.A), 25 g/L urea, 1.1 g/L of creatinine (Sigma) and 0.3% glucose, pH 5.8; (iii) SDL – Sabouraud dextrose liquid medium – dextrose 20.0 g/L, casein 5 g/L, peptic digest of animal tissue 5 g/L, pH 5.6; (iv) YPD – 1% yeast extract, 2% soybean peptone and 2% glucose, pH 6.0 (when cultured under anaerobic conditions in this medium - AN). After subsequent harvesting of 5x10⁹ cells by centrifugation (5 minutes, 3000 g), cell surface shaving with trypsin was performed, followed by the further identification of proteins based on the identification of released peptides by LC-MS/MS using a Dionex Ultimate 3000 UHPLC system coupled to an HCTUltra ETDII mass spectrometer, as described previously in detail (Karkowska-Kuleta *et al.*, 2019). The relative percentage of enolase was calculated after the NSAfs (normalized spectral abundance factors) were determined for each protein identified in the mixture (the spectral counts (SpC) of a protein divided by its length (L); this value is then divided by the sum of all SpC/L) (Zybailov *et al.*, 2005; Zybailov *et al.*, 2007).

4.5. Analysis of the impact of fungal cell environment on the surface localization of *C.*

***albicans* enolase**

C. albicans cells were grown in YPD for 16 hours at 30°C with the addition of 100 ng/mL lipopolysaccharide (LPS; Sigma), 1 mM hydrogen peroxide (Sigma), 200 µM farnesol (F) (trans,trans-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol; Sigma); 200 µM farnesoic acid (FA) (Echelon Biosciences Inc, Salt Lake City, UT), or 2 µM autoinducer-2 (AI-2) ((S)-4,5-dihydroxy-2,3-pentanedione; Biosynth, Staad, Switzerland). Cells grown only in YPD

medium served as a control. After culturing, the fungal cells (2×10^7) were centrifuged (3000 g, 5 minutes) and washed three times with PBS buffer. Then, the immunodetection of surface-exposed enolase was performed as described above with the use Alexa Fluor 488 conjugated secondary antibodies and subsequently the flow cytometry analysis measurements were carried out on a BD LSRFortessa™ cell analyzer (Franklin Lakes, NJ) with an excitation wavelength of 488 nm and an emission wavelength of 519 nm. The values obtained for cells incubated only with secondary antibodies were subtracted from the values obtained for the tested samples.

4.6. Isolation of *C. albicans* cell surface-exposed proteins and purification of Als3 protein

Fungal cell wall-associated proteins were isolated as previously described (Karkowska-Kuleta *et al.*, 2017) with 2 U of β -1,6-glucanase (Takara Bio Inc.) per 0.4 g of *C. albicans* cells (wet weight) that had been grown in filamentous form for 72 hours in RPMI 1640 medium at 37°C. After incubation of hyphae with the enzyme in 1 mL of McIlvaine buffer for 24 hours at 37°C with gentle shaking, the integrity of the cell membranes was confirmed by Trypan Blue (Sigma) staining and the supernatant containing isolated proteins was collected and dialyzed against PBS buffer for 48 hours at 4°C. Als3 protein was purified from the obtained protein mixture with the use of ion-exchange chromatography on a Resource™ Q column (GE Healthcare, Uppsala, Sweden) followed by gel filtration on a Superdex 200 HR column (GE Healthcare/Amersham Biosciences, Little Chalfont, UK) in accordance with a previously described method (Seweryn *et al.*, 2015). Protein concentrations were determined using the Bradford method (Bradford, 1976). The quality and purity of the obtained preparations were verified by SDS-PAGE electrophoresis in the Laemmli system, and the accuracy of

protein purification was assessed using LC-MS/MS, as described earlier (Seweryn *et al.*, 2015).

4.7. Purification of cytoplasmic enolase

C. albicans cytoplasmic enolase was purified as previously described (Seweryn *et al.*, 2015) in accordance with the method of Ballantyne and Warmington, 2000, with minor modifications (Ballantyne & Warmington, 2000). Briefly, *C. albicans* cells grown in YPD medium at 30°C were washed twice with PBS buffer and then subjected to cell breakage in ice-cold water with protease inhibitors (Complete EDTA-free, Roche, Penzberg, Germany) by sonication (10 sets of 1 minute, interval 0.8) with a Sonic Ruptor 400 Ultrasonic Homogenizer (Omni International, Kennesaw GA). After removing the damaged cells by centrifugation (21000 *g*, 30 minutes), solid ammonium sulphate was added to the supernatant to a final saturation of 65%, and then to 100% after another centrifugation step and discarding the precipitant. A protein precipitate obtained at this stage was dissolved and dialyzed against 20 mM Bis-Tris buffer, pH 6.4 and applied to a Mono Q column (Pharmacia Biotech, Piscataway, NJ) and then to a MonoS HR 5/5 column (Pharmacia Biotech) after dialysis of enolase-containing fractions to 10 mM sodium acetate buffer, pH 4.8. Elution for both chromatographic steps was performed in the appropriate buffer using an 80 mL gradient of 0–1 M NaCl at a flow rate of 1 mL/min. *C. albicans* enolase was identified in the collected eluates using SDS-PAGE and protein identification with LC-MS/MS, as described earlier (Seweryn *et al.*, 2015).

4.8. Binding of biotinylated enolase to the fungal cell surface

C. albicans was cultured in liquid YPD medium for 18 hours at 30°C, whereas *S. cerevisiae* wild and mutant strains were cultured in liquid CSM medium for 48 hours at 30°C. Optical

densities were then measured at 600 nm. Culture aliquots were transferred to Eppendorf tubes (1×10^7 *C. albicans* cells and 3×10^7 *S. cerevisiae* cells) or, in the case of *C. albicans* only, to the wells of a Maxisorp microplate (1×10^6) and additionally grown in RPMI 1640 medium at 37°C for 3 hours to obtain filamentous forms. After each step, cells were washed three times with 200 μ L of PBS buffer, pH 7.4, and cells in suspension were centrifuged each time at 3000 *g* for 3 minutes. To avoid any non-specific binding the unoccupied surface in the wells of the microplate was blocked with 300 μ L of 1% BSA for 1 hour at 37°C and the cells in the Eppendorf tubes were transferred to new tubes just prior to the detection of bound protein. 50 μ L of *C. albicans* enolase (Eno-Bt), biotinylated using biotin N-hydroxysuccinimide ester (Sigma) according to a previously described procedure (Rapala-Kozik *et al.*, 2008), was added to the cells at a concentration range of 25-200 nM for *C. albicans* and 6.25-75 nM or only 250 nM for *S. cerevisiae*, and incubated for 1 hour at 37°C. After washing out unbound protein, 50 μ L of horseradish peroxidase-conjugated streptavidin (SA-HRP, MP Biomedicals) diluted 1:4000 in PBS was incubated with fungal cells for 1 hour at room temperature in the dark. The amount of bound Eno-Bt was measured with the addition to the cells of 50 μ L of substrate for HRP, TMB (Sigma) and the reaction was stopped after 2 minutes with 50 μ L of 2 M HCl. After centrifugation, 90 μ L of the supernatant was transferred into wells of a standard 96-well microtiter plate (Sarsted, Germany) and the absorbance at 450 nm was measured in a Power Wave X Select microplate reader (BioTek Instruments). The control readings were the values calculated for the yeast-like or hyphal forms incubated without enolase. To compare the relative fusion protein expression level at the surface of *S. cerevisiae* mutant strains, 3×10^7 yeast cells were transferred to Eppendorf tubes and incubated with 50 μ L of a 1 μ g/mL solution of mouse anti-HA antibody (ZYMED Laboratories) in PBS for 1 hour at 37°C, washed to remove

unbound primary antibodies, and further incubated with 50 μ L of secondary anti-mouse antibodies (1 μ g/mL) conjugated with HRP. The cells were then transferred to new tubes, and the reaction was developed with 50 μ L TMB for 40 sec, stopped with 50 μ L of 2 M HCl, and after cell removal by centrifugation (3000 *g*, 5 minutes), 90 μ L of supernatant was transferred to the microplate, and the absorbance was measured as described above.

A similar procedure was used for microscopic observations, however, enolase was labelled with NHS-fluorescein (1 mg per 100 μ L of dimethylformamide; Sigma) in 0.1 M carbonate buffer at pH 8.3 at 4°C for 2 hours and then dialyzed against PBS, pH 7.4 at 4°C for 48 hours. *C. albicans* hyphal forms, as described above, were grown in the wells of a glass bottom 96-well microplate (CellVis), incubated with enolase for 1 hour at 37°C. Imaging was performed using an Olympus IX73 microscope.

4.9. Identification of *C. albicans* enolase-binding cell wall proteins with the use of affinity chromatography and chemical cross-linking

Affinity chromatography and chemical cross-linking were performed as described previously in detail (Kozik *et al.*, 2015). Briefly, an affinity gel with covalently conjugated enolase was prepared via the immobilization of 100 μ g enolase in 0.1 M HEPES buffer, 80 mM CaCl_2 , pH 7.5 to 1 mL of Affi-Gel 10 (Bio-Rad, Herkules, CA). The *C. albicans* cell wall proteins (250 μ g) isolated with β -1,6-glucanase were incubated at 37°C for 4 hours with 50 μ L of the gel. After then removing unbound proteins by extensive washing with 1 mL of PBS buffer, bound proteins were eluted with 2% SDS at 95°C for 30 minutes. Proteins eluted from a gel without immobilized enolase but incubated with the mixture of *C. albicans* proteins were used as a control for “non-specific” binding. Additionally, the chemical cross-linking of the biotinylated enolase (10 μ g in 100 μ L PBS, pH 7.4) and β -1,6-glucanase-

extractable *C. albicans* cell wall proteins (250 µg) was performed using 0.5 mM sulfosuccinimidyl 2-([4,4'-azipentanamido]ethyl)-1,3'-dithiopropionate (sulfo-SDAD) (Thermo Fisher Scientific). Covalently linked pairs of biotinylated enolase and other fungal cell wall proteins were adsorbed on MagnaBind streptavidin beads (Thermo Fisher Scientific) and released after washing out unbound proteins by treatment with 2.5% β-mercaptoethanol and 2% SDS for 30 minutes. Proteins eluted after incubation of streptavidin beads alone with non-biotinylated proteins were used as a "non-specific" binding control. Proteins eluted after affinity chromatography were separated by SDS-PAGE and bands that were not present in the control samples were excised and identified using LC-MS/MS as described above.

4.10. Characterization of the Als3 interaction with *C. albicans* enolase using SPR measurements

The kinetic and thermodynamic analysis of *C. albicans* Als3 binding to enolase was performed using a BIACORE 3000 system (GE Healthcare, Uppsala, Sweden). After dialysis in 10 mM HEPES buffer, pH 7.4, Als3 was immobilized onto a CM3 sensor chip surface (GE Healthcare), using the Amine Coupling Kit (GE Healthcare). After activation of the chip surface with 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 200 mM N-hydroxysuccinimide (NHS), Als3 in 10 mM sodium acetate buffer (pH 4.0) was injected over the chip surface at a flow rate of 10 µL/min. The obtained immobilization level reached 90 resonance units (RU). The running buffer - 10 mM HEPES with 150 mM NaCl and 0.005% surfactant P20 (pH 7.4) - and the solutions of enolase (in the concentration range of 5-100 nM) in the same buffer were injected at a flow rate of 30 µL/min at 25°C. The association and dissociation time was 180 seconds. The injection of 1 M NaCl at a flow rate of 30 µL/min

for 30 seconds was used as a regeneration step. The obtained results were analyzed using BIAevaluation 4.1 software (GE Healthcare). The equilibrium dissociation constant (K_D) was calculated by fitting a homologous model that separately determines the dissociation and association rate constants (k_d and k_a).

4.11. Analysis of the *C. albicans* cell surface interaction with fungal enolase and human proteins

Flow cytometry measurements were carried out on a BD LSRFortessa™ cell analyzer (Franklin Lakes, NJ) and with the *S. cerevisiae* strain UB2316. Aliquots of 5×10^6 yeast cells were transferred into Eppendorf tubes, suspended in 500 μ L PBS buffer, and centrifuged for 3 minutes (3000 *g*). Human proteins—HK and HPG—were labelled with NHS-fluorescein (1 mg per 100 μ L of dimethylformamide; Sigma) in 0.1 M carbonate buffer at pH 8.3 at 4°C for 2 hours and enolase with a 30-molar excess of Alexa Fluor 647 (Thermo Fisher Scientific) solution prepared in dimethyl sulfoxide (DMSO) at 37°C for 1 hour. The labelled protein was then dialyzed against PBS, pH 7.4 at 4°C for 48 hours after which the protein concentration was tested using a NanoDrop spectrophotometer. Yeast enolase conjugated with Alexa Fluor 647 was added to cell pellets at increasing concentrations (13.3 nM -1700 nM) together with fluorescein-conjugated HPG or HK at two concentrations (25 nM and 50 nM). Yeast cells with added proteins were incubated for 90 minutes at 37°C and the cells were then washed three times with PBS buffer and 100 μ L of paraformaldehyde was added for 15 minutes. The cells were then transferred to glass tubes for flow cytometric analysis and placed at 4°C. On the following day, the samples were subjected to fluorescence analysis at excitation wavelengths of 488 and 633 nm and emission wavelengths of 519 and 660 nm for fluorescein and Alexa Fluor 647-labels, respectively.

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Supplementary Materials: Supplementary Table 1. Mass spectrometry identification of *C. albicans* proteins present at the cell surface under different growth conditions.

Supplementary Table 2. Mass spectrometric parameters for identification of putative enolase-binding *C. albicans* cell wall-associated proteins. Supplementary figures:

Supplementary figure S1. *C. albicans* growth curve (up to 48 h) and the detection of enolase at the surface of the yeast cells. Supplementary figure S2. Detection of surface-located enolase on *C. albicans* cells correlated with morphological changes observed during cell growing at increased temperature (30°C versus 37°C). Supplementary figure S3. Stimulation of enolase surface display in *C. albicans* cells growing in the YPD medium supplemented with selected host proteins. Supplementary figure S4. Comparison of the surface of *C. albicans* cells growing in different morphological forms. Supplementary figure S5. The plot of response at $R_{\text{equilibrium}}$ (R_{eq}) against the enolase concentration (SPR analysis) presenting the saturation of the binding sites of ligand by the analyte.

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Data availability: The main data that supports the findings of this study are available in the article and in the supplementary material of this article. The detailed data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Identification of putative enolase-binding candidal cell wall-associated proteins.

C. albicans cell wall-associated proteins were isolated from germ tubes and hyphal forms with β -1,6-glucanase. Putative enolase-binding proteins were isolated from protein mixtures via affinity chromatography (AC) or identified by chemical cross-linking (CL). The number of plus signs corresponds to the frequency with which the protein was identified by the given method across four biological replicates.

ENTRY (ENTRY NAME)	PROTEIN NAME	AC	CL
Q59L12 (ALS3_CANAL)	Agglutinin-like sequence protein 3	++++	++++
P40953 (CHI2_CANAL)	Chitinase 2	+	
O13287 (6PGD_CANAX)	6-Phosphogluconate dehydrogenase	+	++
P30575 (ENO1_CANAL)	Enolase		++++
P83780 (G6PI_CANAL)	Glucose-6-phosphate isomerase		++++
P82612 (PMGY_CANAL)	Phosphoglycerate mutase		++

Table 2. *S. cerevisiae* strains used in this study.

Strain	Description	Reference
<i>Saccharomyces cerevisiae</i> BY4742 UB2022	<i>MATα leu2Δ0 lys2Δ0 ura3Δ0 his3Δ1</i>	(Brachmann <i>et al.</i> , 1998)
UB2155	BY4742 with pBC542 (9.2 kb; ApR; pMB1 <i>ori</i> ; <i>URA3</i>)	(Nobbs <i>et al.</i> , 2010)
UB2157	BY4742 with pBC542- <i>ALS3lg</i>	(Nobbs <i>et al.</i> , 2010)
UB2158	BY4742 with pBC542- <i>EAP1</i>	(Nobbs <i>et al.</i> , 2010)
UB2159	BY4742 with pBC542- <i>HWP1</i>	(Nobbs <i>et al.</i> , 2010)
UB2161	BY4742 with pBC542- <i>CWP1</i>	(Nobbs <i>et al.</i> , 2010)
UB2307	BY4742 with pBC542- <i>ALS3Δ218-285</i>	(Bamford <i>et al.</i> , 2015)
UB2316	BY4742 with pBC542- <i>ALS3Δ325-331</i>	(Bamford <i>et al.</i> , 2015)
UB2418	BY4742 with pBC542- <i>ALS3Δ166-225</i>	(Bamford <i>et al.</i> , 2015)
UB2419	BY4742 with pBC542- <i>ALS3Δ270-305</i>	(Bamford <i>et al.</i> , 2015)
UB2420	BY4742 with pBC542- <i>ALS3Δ277-286</i>	(Bamford <i>et al.</i> , 2015)
UB2421	BY4742 with pBC542- <i>ALS3Δ434-830</i>	(Bamford <i>et al.</i> , 2015)

Take Away

- *C. albicans* enolase is exposed on the cell surface in different conditions
- Greater enolase abundance was found at the surface of fungal filamentous forms
- Candidal surface adhesin Als3 is involved in the binding of enolase
- N-terminal and central repeat domains of Als3 are crucial for enolase display
- Surface enolase regulates Als3 interactions with plasminogen and kininogen

For Peer Review

Figure legends

Figure 1. *C. albicans* growth curve and the detection of enolase at the surface of the yeast cells. Aliquots of 2×10^6 *C. albicans* yeast cells were placed into the wells of a 96-well microtiter plate in 200 μ L of YPD medium for 19 hours. The cell growth was followed by OD measurements at 600 nm, whereas cell viability was analyzed with XTT tests at specified time intervals. The presence of enolase at the cell surface was detected using mouse monoclonal anti-enolase antibodies (1 μ g/mL) in PBS and goat anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP). Signals were detected using TMB as a substrate for HRP.

Figure 2. Display of enolase on the surface of *C. albicans* cells growing under different environmental conditions. (A) *C. albicans* cells were cultured aerobically in various media (AU – artificial urine, AS – artificial saliva, SDL – Sabouraud dextrose liquid medium) or cultured anaerobically in YPD medium (AN). Surface-exposed proteins were identified by cell surface shaving with trypsin followed by LC-MS/MS. The relative percentage of enolase was calculated after the mathematical normalization of spectral abundance factors. The sum of the normalized spectral abundance factor values calculated for all proteins identified in a given sample was taken as 100%. Bars represent the mean values of 3 determinations $\pm$ standard deviation (SD). (B) *C. albicans* cells were cultured in YPD medium without (C, control) or with one of the indicated supplements (farnesoic acid (FA) (200 μ M), farnesol (F) (200 μ M), autoinducer-2 (AI-2) (2 μ M), lipopolysaccharide from *Escherichia coli* (LPS) (100 ng/mL) or H_2O_2 (1 mM)) for 16 hours at 30°C. Cells were incubated with mouse anti-enolase antibodies and subsequently with secondary anti-mouse antibodies conjugated with Alexa Fluor 488. After gentle cell washing, flow cytometry analysis was

performed, with fluorometric detection conducted at an excitation wavelength of 488 nm and emission wavelength 519 nm. The control measurements (the values for cells incubated only with secondary antibodies) were subtracted from the total binding values. Data are plotted as means $\pm$ standard error of the mean. Statistical significance levels were determined using one-way ANOVA with Dunnett's multiple comparisons test and are marked relative to the amount of enolase exposed in the YPD medium; **** $p < 0.0001$.

Figure 3. Differences in the enolase levels at the surface of *C. albicans* cells induced to grow as different morphological forms. *C. albicans* cells were grown for 16 hours in YPD medium at 30°C (blastospores) or in RPMI 1640 for 3 hours at 37°C (filamentous forms). (A, B) The amount of surface-exposed enolase was detected with mouse monoclonal anti-enolase specific antibodies and Alexa Fluor 488 labeled anti-mouse secondary antibodies. The measured parameter was the mean fluorescence intensity, which was determined as the ratio of the fluorescence intensity presented by the single cell to its surface area; (C) blastospores (10^7 cells) and filamentous forms (obtained from 10^6 yeast-like cells incubated in wells of MaxiSorp microplates) of *C. albicans* were incubated with mouse monoclonal anti-enolase antibodies and anti-mouse antibodies conjugated with HRP, and the detection was performed using TMB as a HRP substrate with the absorbance measurement at 450 nm. The control readings (the values calculated for the yeast-like or filamentous forms incubated only with HRP-conjugated secondary antibodies) were subtracted from the total binding values. Data are plotted as means from three determinations $\pm$ standard error of the mean. Statistical significance levels were determined using a Student t-test and are marked relative to each other with ** for $p < 0.01$.

Figure 4. Binding of enolase to the surface of the yeast-like and filamentous forms of *C.*

***albicans* cells.** (A) Blastospores (10^7 cells) and the filamentous forms (obtained from 10^6 yeast-like cells incubated for 3 hours at 37°C in 100 µL RPMI 1640 medium in wells of MaxiSorp microplates) of *C. albicans* were incubated with 50 µL biotin-labeled purified cytosolic *C. albicans* enolase (25-200 nM) for 1 hour at 37°C in PBS buffer, pH 7.4. The bound protein levels were determined using the SA-HRP/TMB detection system and the absorbance was measured at 450 nm. The control readings (the values calculated for the yeast-like or hyphal forms incubated without enolase) were subtracted from the total binding values. Data are plotted as means from three determinations $\pm$ standard error of the mean. Statistical significance levels were determined using a Student t-test and are marked with * for $p < 0.05$ and ** for $p < 0.01$. (B) *C. albicans* cells were grown in RPMI 1640 for 3 hours at 37°C to obtain hyphal forms and then were incubated with 50 µL fluorescein-labeled purified cytosolic *C. albicans* enolase for 1 hour at 37°C in PBS buffer, pH 7.4.

Figure 5. Binding of *C. albicans* enolase to *S. cerevisiae* cells harboring surface-expressed

candidal adhesins. *S. cerevisiae* cells (3×10^7) expressing the *C. albicans* adhesins Als3, Eap1, and Hwp1, a control protein Cwp1, and pBC542 vector alone, were incubated with 50 µL of *C. albicans* biotin-labeled enolase in PBS buffer, pH 7.4 for 1.5 h at 37°C with gentle stirring. The amount of bound protein was determined using the SA-HRP/TMB detection system, and the absorbance was measured at 450 nm. The values for the control strain harboring the pBC542 vector alone were subtracted from the other values. The experiment was performed in triplicate and cumulative results presented with data points reported as the means $\pm$ SEM. Binding curves representing data fits with nonlinear regression were generated with GraphPad Prism software.

Figure 6. SPR analysis of the Als3-enolase interaction. Sensograms were obtained after injection of enolase at a concentration range of 5–100 nM and at a flow rate of 30 μ L/min over a CM3 chip containing Als3 immobilized to a level of 90 RU. The binding parameters for the interactions between these fungal proteins were determined after fitting the data illustrated by a black line and are presented with the standard error (SE). The graph illustrating the equilibrium responses (Req) as a function of enolase concentration (up to 400 nM) is presented in the Supplementary materials (Figure S5).

Figure 7. Mapping of the Als3 regions involved in enolase binding. (A) schematic representation of Als3 expressed on the surface of *S. cerevisiae* and corresponding deletion mutants, based on (Bamford *et al.*, 2015), AFR – amyloid-forming region, GPI – glycosylphosphatidylinositol, HA – hemagglutinin; (B) *S. cerevisiae* cells (3×10^7 cells) expressing *C. albicans* adhesin Als3, specific deletion mutants of Als3, or the pBC542 vector alone, were incubated with *C. albicans* biotin-labeled enolase. The amount of bound protein was determined with the SA-HRP/TMB detection system. The values obtained with the vector alone control were subtracted from all other values. The experiment was performed in triplicate and a representative result is shown as the mean $\pm$ standard deviation (SD). Statistical significance levels were determined using one-way ANOVA with a Tukey's multiple comparisons test relative to pBC-Als3; ** $p < 0.01$, *** $p < 0.001$.

Figure 8. Characteristics of the mutual interactions between the HK and HPG host proteins and Als3 and Eno1 on the yeast cell surface. (A-B, E-F) *S. cerevisiae* cells (5×10^6 cells per point) expressing surface Als3 lacking its amyloid fragment were incubated in suspension (50 μ L) with HPG or HK host proteins conjugated with fluorescein and over an increasing concentration range of enolase (Eno1) conjugated with Alexa Fluor 647. After 90

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minutes of incubation and gentle cell washing, flow cytometry analysis was performed, with fluorometric detection conducted at excitation wavelengths of 488 nm or 633 nm and emission wavelengths 519 nm or 660 nm for fluorescein or Alexa Fluor 647, respectively.

(C) The binding of the HK and HPG in the presence of Eno1 was analyzed in the same *S. cerevisiae* model using biotinylated host proteins and the SA-HRP/TMB detection system.

(D) Identification of the Als3 regions important for these mutual interactions was performed using the *S. cerevisiae* surrogate for Als3 as described above and the SA-HRP/TMB detection system.

For Peer Review

**The Als3-mediated attachment of enolase on the surface of *Candida albicans* cells
regulates their interactions with host proteins**

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Running title: Als3-mediated surface location of *C. albicans* Eno1

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Summary

The multifunctional protein enolase has repeatedly been identified at the surface of numerous cell types, including a variety of pathogenic microorganisms. In *Candida albicans*—one of the most common fungal pathogens in humans—a surface-exposed enolase form has been previously demonstrated to play an important role in candidal pathogenicity. In our current study, the presence of enolase at the fungal cell surface under different growth conditions was summarized and a higher abundance of enolase at the surface of *C. albicans* hyphal forms compared to yeast-like cells was found. Affinity chromatography and chemical cross-linking indicated a member of the agglutinin-like sequence protein family—Als3—as an important potential partner required for the surface display of enolase. Analysis of *Saccharomyces cerevisiae* cells overexpressing Als3 with site-specific deletions showed that the Ig-like N-terminal region of Als3 (aa 166-225; aa 218-285; aa 270-305; aa 277-286) and central repeat domain (aa 434–830) are essential for the interaction of this adhesin with enolase. In addition, binding between enolase and Als3 influenced subsequent docking of host plasma proteins—high molecular mass kininogen and plasminogen—on the candidal cell surface, thus supporting the hypothesis that *C. albicans* can modulate plasma proteolytic cascades to affect homeostasis within the host and propagate inflammation during infection.

1. Introduction

Yeast-like fungus *Candida albicans* is among the common commensal colonizers of the human skin, vagina, oral cavity and gut, but can also initiate the development of a wide range of diseases in patients with impaired immunity. There is currently a broad clinical spectrum of these disorders, candidiases, ranging from mucocutaneous overgrowth to disseminated infections like candidemia (Lim, Rosli, Seow, Chong, 2012). The pathogenesis of *C. albicans* infections is mediated by several virulence factors and mechanisms, including the morphological transition from yeast-like to hypha-forming cells and phenotypic switching (i.e. the white-to-opaque transition). Both mechanisms facilitate adaptation of *C. albicans* to the changing environmental conditions encountered during colonization of new niches within the host. The main virulence factors of *C. albicans* include secreted hydrolytic enzymes, mainly phospholipases and proteinases, and the cytolytic peptide toxin—candidalysin that enable evasion of antimicrobial host defenses and contribute to pathogenesis by disruption of host cell membranes or degradation of host immune proteins or peptides (Naglik, Challacombe, Hube, 2003; Moyes *et al.*, 2016).

One of the key features of candidiasis development is the adhesion of *C. albicans* to human epithelial or endothelial cells, during which both morphotypes can engage several adhesins and other surface-located proteins. Moreover, the adhesive properties of fungal cells facilitate the formation of biofilms, which promote persistent host colonization and increased resistance to host defenses or antimycotic treatments (Tsui, Kong, Jabra-Rizk, 2016). Among the adhesins identified on the *C. albicans* cell surface, members of the agglutinin-like sequence (Als) protein family (Als1-9) play an essential role in establishing *C. albicans*-host interactions (Hoyer & Cota, 2016). These proteins are glycosylphosphatidylinositol (GPI)-linked to β -1,6-glucans of the fungal cell wall (Gaur &

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3 Klotz, 1997; Hoyer, 2001; Sheppard *et al.*, 2004); some are preferentially expressed by germ
4 tubes and hyphae (Als1-4), and others by yeast-like cells (Als5-7, Als9) (Modrzewska &
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6 Kurnatowski, 2015). Proteins Als2, Als3, and Als4 have been identified as facilitating *C.*
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8 *albicans* adhesion to host cells and, for Als3, E-cadherin on epithelial cells or N-cadherin on
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10 endothelial cells were shown to be binding targets (Phan *et al.*, 2007). Host extracellular
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12 matrix (ECM) proteins, including fibronectin, laminin, collagen, gp96, EGFR and HER2, have
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14 also been identified as Als binding partners. Additionally, specific proteins of the blood
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16 coagulation cascade such as kininogen, plasminogen and fibrinogen were found to bind Als3
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18 (Gaur, Smith, Klotz, 2002; Gaur and Klotz, 2004; Sheppard *et al.*, 2004; Phan *et al.*, 2007;
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20 Almeida *et al.*, 2008; Silverman, Nobbs, Vickerman, Barbour, Jenkinson, 2010; Karkowska-
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22 Kuleta *et al.*, 2011; Liu, Mittal, Solis, Prasadaraao, Filler, 2011; Zhu *et al.*, 2012; Seweryn *et*
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24 *al.*, 2015).

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33 Alongside dedicated adhesins such as the Als family, the *C. albicans* cell surface is
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35 equipped with so-called “moonlighting” proteins. These are abundantly expressed at the
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37 cell surface but are not secreted via the classical secretory pathways. These factors include
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39 chaperones, stress protectants, and enzymes involved in intracellular processes such as
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41 glycolysis and gluconeogenesis, the citric acid cycle, the pentose phosphate pathway, and
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43 the metabolism of DNA and proteins (Karkowska-Kuleta & Kozik, 2014; Jeffery, 2018;
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45 Karkowska-Kuleta, Satala, Bochenska, Rapala-Kozik & Kozik, 2019). However, when
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47 associated with the cell surface, these moonlighting proteins seem to perform alternative
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49 functions. Several bacteria and fungi have been found to use moonlighting proteins as
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51 receptors for host proteins, mediating physical attachment to the host cell surface or
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53 facilitating invasion, virulence, biofilm development or disseminated infection (Satala,
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55 Karkowska-Kuleta, Zelazna, Rapala-Kozik, Kozik, 2020a).

One of the most crucial moonlighting proteins of *C. albicans* is enolase, an evolutionarily conserved enzyme of the glycolysis and gluconeogenesis pathways. Its extracellular function is important for infection propagation and seems to be universal amongst different fungal species, bacteria, and parasites (Jeffery, Bahnson, Chien, Ringe, Petsko, 2000; Ehinger, Schubert, Bergmann, Hammerschmidt, Heinz, 2004; Liu *et al.*, 2012; Chen, Zabad, Liu, Wang, Jeffery C, 2018; Karkowska-Kuleta *et al.*, 2018). These proteins present a high degree of sequence homology between enolases of evolutionarily distant organisms, including mammals (Hannaert *et al.*, 2003; Macías-Sánchez *et al.*, 2015).

Enolase also displays multiple functions within the cell. In the cytoplasm of mammalian, fungal and bacterial cells, enolase functions as a chaperone, and was previously proposed to be involved in the survival and proliferation of *Streptococcus mutans* at low pH (Wilkins, Homer, Beighton, 2002), *Escherichia coli* under oxidative stress (Tamarit, Cabiscol, Ros, 1998), and various yeasts under heat shock (Iida & Yahara, 1985). Previous studies also indicated an association between enolase and the mitochondrial membrane, and it has been ascribed as a RNA chaperone due to RNA binding activity (Brandina *et al.*, 2006; Entelis *et al.*, 2006). Enolase was also recognized as a constituent and regulator of vacuoles, especially in *Plasmodium falciparum*, where different Eno variants were involved in parasite survival in a hostile environment via the complex neutralization of free heme (Shevade, Jindal, Dutta, Jarori, 2013). In the yeast *S. cerevisiae*, both Eno1 and Eno2 proteins stimulate the fusion of vacuoles and probably perform a transporter function (Decker & Wickner, 2006; Wiederhold *et al.*, 2009).

Eno1 exposed at the *C. albicans* cell surface was involved in the binding of human plasminogen (Crowe *et al.*, 2003). It has also been found to interact with plasma contact factors: kininogen, factor XII and prekallikrein, leading to host-independent production of

kinins—the vasoactive peptides conducive to the spread of yeast infection (Seweryn *et al.*, 2015; Funk *et al.*, 2016). Several studies have implicated enolase as protein mediating microbial adhesion to host extracellular matrix proteins such as fibronectin, laminin and vitronectin (Carneiro, Postol, Nomizo, Reis, Brentani, 2004; Esgleas *et al.*, 2008; Castaldo *et al.*, 2009; Donofrio *et al.*, 2009; Satala *et al.*, 2020b) and promoting a fast humoral response in patients with systemic candidiasis (Strockbine, Largen, Zweibel, Buckley, 1984). Enolase was also proposed to be an important allergen in lung diseases (Ito, Ishiguro, Kanbe, Tanaka, Torii, 1995). Moreover, being commonly identified in the blood of infected patients, *C. albicans* enolase is used as a marker of invasive candidiasis (Walsh *et al.*, 1991). Unexpectedly, enolase localized on the *C. albicans* cell surface also shows transglutaminase activity, contributing to cell morphological changes and cell wall remodeling, but its exact role in the pathogenicity of these fungi is not fully understood (Reyna-Beltrán *et al.*, 2018).

C. albicans enolase has been detected on the surface of both blastospores and hypha, where its association with cell wall glucans was proposed (Angiolella *et al.*, 1996). Although little is known about the transport of enolase to the fungal cell surface, studies of post-translational modifications that can occur during this process for human and bacterial enolases have been reported (Boël *et al.*, 2004; Daubenspeck, Liu, Dybvig, 2016; Zakrzewicz *et al.*, 2018). It has also been shown that enolase can be secreted outside the *C. albicans* cells as a cargo in extracellular vesicles (Gil-Bona *et al.*, 2015; Vargas *et al.*, 2015; Wolf, Espadas, Luque-Garcia, Reynolds, Casadevall, 2015). Silva and coworkers (2014) documented the presence of enolase within biofilm structures and demonstrated that this did not arise from cell lysis and subsequent protein readsorption to the cell surface. Their analyses also showed that blocking of cell surface enolase by protein-specific antibodies inhibited the adherence of *C. albicans* cells to mice GI tissue (Silva *et al.*, 2014).

Given the limited understanding of the regulation and role of enolase at the fungal cell surface this study aimed to analyze enolase exposure at the *C. albicans* cell surface under different growth conditions and the potential for readsorption of cytosolic enolase. The role of external enolase in mediating the interactions of fungal cells with host plasma proteins involved in the blood coagulation/fibrinolysis cascades, human plasminogen (HPG) and human high molecular mass kininogen (HK), was also experimentally addressed.

2. Results

2.1. Enolase is exposed on the C. albicans yeast-like cell surface during the stationary phase of growth

The origin of the enolase that has been repeatedly identified at the surface of *C. albicans* yeast-like cells, hyphae, and biofilm-forming cells (Pancholi, 2001; Silva *et al.*, 2014) still remains controversial. Hence, in our present study, we conducted a stepwise analysis of the surface appearance of enolase, using *C. albicans* strain ATCC10231 grown in YPD medium at 30°C. Under these growth conditions, the cells were in a yeast-like form and did not form hyphae. The progress in cell growth was determined by optical density measurements carried out concurrently for 19 hours with a determination of cell viability using the XTT colorimetric assay for cellular metabolic activity (Figure 1). The appearance of enolase at the *C. albicans* cell surface was detected with a specific antibody and reached its highest level after 19 hours of growth under the conditions studied, in which the cells had reached the stationary growth phase. Monitoring of enolase levels over the longer time scale showed no significant changes in the amount of this protein, which remained almost constant on the surface of *C. albicans* cells (Figure S1 in Supplementary materials).

2.2. The fungal cell environment influences the surface localization of enolase

2.2.1. Enolase display at the candidal cell surface depending on the growth conditions

As the environment around *C. albicans* cells may affect the yeast metabolism and physiology, we evaluated the possible modulation of enolase display at the fungal cell surface at variable conditions of cell growth. For the detection of *C. albicans* surface-displayed enolase after culture in different media in shaking flask cultures, where cells are more likely to grow in a planktonic form, cell surface shaving with trypsin and liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) were used (Figure 2A; mass spectrometric details for protein identification are included in Supplementary Table 1). This method of identifying proteins and estimating their relative abundance at the cell surface from the calculation of the normalized spectral abundance factors (NSAFs) (Zybailov, Coleman, Florens and Washburn 2005; Zybailov, Florens, and Washburn 2007) has been successfully used previously for *C. albicans* cells (Gil-Bona *et al.*, 2015; Marín *et al.*, 2015). Relative enolase display at the surface of the fungal cells grown in different media was compared with cells propagated aerobically in YPD medium, which is commonly and routinely used for yeast culture. Some of the tested growth media simulated roughly the conditions that prevail in different infectious niches such as artificial saliva (AS) and artificial urine (AU), thus providing some approximation of the conditions under which infections occur in the oral cavity or urinary tract, respectively. In addition, enolase exposure during growth under anaerobic conditions in YPD medium was also tested (AN).

Even considering that the detection of enolase was semi-quantitative, this protein was identified at comparable levels on the surfaces of *C. albicans* cells cultured under very different conditions, suggesting that its surface location is a general phenomenon. The

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3 highest level of changes in the surface presence of enolase was noted for medium SDL,
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5 containing casein. However, as that was a relative measurement, it is possible to some
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7 degree that it may reflect not only the accumulation of enolase at the cell surface but,
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9 additionally, the loss of other surface proteins. A complete list of proteins for each culture
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11 condition is presented in Supplementary Table 1.
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15 *C. albicans* cells growing at the place of infection in mono-species or multi-species
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17 consortia prefer to form a biofilm regulated by quorum sensing molecules (QSM). Using
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19 immunodetection and flow cytometry, we tested whether the process of enolase **display** at
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21 the surface of *C. albicans* cells could be affected by the fungal or bacterial quorum-sensing
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23 molecules, farnesol (F) and farnesoic acid (FA), the autoinducer-2 (AI-2), or the major
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25 lipopolysaccharide (LPS) component of the outer membrane of Gram-negative bacteria that
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27 fungi may come into contact with during the creation of a mixed-species biofilm (Bandara,
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29 Yau, Watt, Jin, Samaranayake, 2009). As the involvement of enolase in response to oxidative
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31 stress was indicated previously (Serrano-Fujarte, López-Romero, Cuéllar-Cruz, 2016), the
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33 effect of exogenous H₂O₂ on enolase presence at the surface of *C. albicans* cells was also
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35 tested (Figure 2B). Enolase was found under all of these conditions at a high but comparable
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37 level to the control cultures of *C. albicans* blastospores grown in YPD medium for 16 hours.
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50 2.2.2. Morphology of *C. albicans* influences the enolase **display** on the fungal cell surface

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52 The presence of enolase at the cell surface was next analyzed during the growth of the
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54 different morphological forms of *C. albicans* cells i.e. yeast-like cells (blastospores) vs.
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56 filamentous forms.
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Since a definitive change in the morphological form usually results from a significant change in growth conditions, we initially estimated the direction of these changes using *C. albicans* cells growing in the Saboraud medium for 16 h, but under different thermal conditions (30°C versus 37°C) that trigger filamentation. The enolase cell surface display was determined using anti-enolase primary antibodies and secondary antibodies conjugated with HRP and TMB as a substrate for HRP. At a temperature of 37°C we observed an increase of enolase presence on the cell surface, correlating with the initiated filamentation (Figure S2 in the Supplementary materials). Another approximation was made with the cells growing in YPD medium enriched with selected plasma proteins at a concentration corresponding to their presence in plasma. As protein content drives *C. albicans* cell filamentation, we observed an accompanying increase in enolase display on the hyphae surface (Figure S3 in the Supplementary materials). In further experiments we used unicellular yeast cells, obtained after growth in YPD medium as described above, whereas the hyphal forms of *C. albicans* cells were produced in microplate wells or on coverslips coated with poly-L-lysine, in RPMI 1640 medium for 3 hours at 37°C. The presence of enolase at the cell surface was then tested microscopically (Figure 3A,B), with specific fluorescently labeled antibodies, or spectroscopically (Figure 3C), using the antibody conjugated with HRP. These two methods have been chosen to account for differences when comparing the surface area of the different morphological forms of fungal cells. The fixation of the cells was omitted to avoid non-specific binding. The fluorescence intensity measurements were calculated per surface area of each cell, determined according to the modified method presented by Urban and co-workers (Hosseinzadeh & Urban, 2013) using the Olympus Cell Sense software (Figure 3B). Similar calculations of cell surface area were made also for the second experimental system where enolase detection was performed with the HRP/TMB system. Taking into

account the calculations carried out for the surface area of both types of cells, in this experiment a 10-fold excess of blastospores was used in relation to the number of cells forming hyphae. The detailed calculations were presented in the Supplementary materials in Figure S4. The amount of surface-located enolase was reported as the absorption of converted TMB substrate (Figure 3C).

In both approaches, greater enolase abundance was found at the surface of hyphal forms, indicating a preference for these morphological forms. This might also be indirectly related to the generally greater abundance of intracellular enolase in hyphal forms than in blastospores—2% vs. 0.7% of the total protein content, respectively, detected by Sundstrom and Aliaga (1994).

2.3. Excreted enolase is re-adsorbed on the surface of the filamentous form of *C. albicans* cells

Since enolase is found at the surface of *C. albicans* cells, although its secretion pathways can be variable and have not yet been identified in detail, such as a possible origin from surrounding dead cells or as a cargo of extracellular vesicles, the possibility of the readsorption of enolase from the outside of the cell to its surface was tested in our current experiments (Figure 4). For this analysis, enolase purified from the cytosol of *C. albicans* cells was employed as it more closely reflects the biological and functional state of this protein. It was previously shown that cytosolic enolase can adsorb to *C. albicans* cell walls that were isolated after breaking the cells with glass beads (Eroles, Sentandreu, Elorza, Sentandreu, 1997). Nevertheless, in our present study we used whole, intact fungal cells,

and found that the association of exogenous enolase was undeniably apparent for both morphological forms of *C. albicans*. We used fluorescein-labeled enolase for microscopic observations or biotin-labeled enolase for the detection with the SA-HRP/TMB system. We further observed an increase in the amount of enolase deposited at the cell surface upon exposure to higher concentrations of added protein. These findings also confirmed that more enolase can be readsorbed to the surface of the hyphal forms of fungal cells than to blastospores. Importantly, this could be related to the change in the proteome of the surface of *C. albicans*, which has been frequently identified in connection with changes in the morphological forms of fungal cells (Martínez-Gomariz *et al.*, 2009; Heilmann *et al.*, 2011). This raises a crucial question as to whether particular proteins located at the surface of *C. albicans* cells are receptors or binding proteins for secreted enolase. We thus next searched for potential proteinous sites for enolase deposition at the fungal cell surface.

2.4. The *C. albicans* surface adhesin Als3 is involved in the binding of enolase

Two complementary methods were used for the isolation and identification of putative enolase-binding proteins from mixtures of proteins extracted from the surface of *C. albicans* cells grown in the form of germ tubes and hyphae. One method involved the use of affinity chromatography with agarose-coupled fungal enolase, whilst the second was based on chemical cross-linking with a heterobifunctional, photoactivated reagent and biotin-labeled *C. albicans* enolase. Only the protein bands that were not present in the control sample were excised from the electrophoretic gel and subjected to in-gel tryptic digestion. The obtained results thus indicate the most frequently identified putative interaction partners for *C. albicans* enolase (Table 1; detailed parameters from mass spectrometry identification are included in Supplementary Table 2). Among the typical cell wall proteins covalently

bound to the fungal cell surface, primarily Als3 was indicated as highly capable of binding to enolase, as it was identified four times, both after using chromatography and cross-linking. Other proteins, which were identified similarly often or with a slightly lower frequency, were, like enolase, atypical cell wall proteins and their presence in the samples may have been related to similar mechanisms of deposition at the cell surface, an affinity for enolase, or a mutual affinity related to the formation of known complexes of several glycolytic enzymes (Kurganov, Sugrobova, Mil'man, 1985; Araiza-Olivera *et al.*, 2013; Menard, Maughan, Vigoreaux, 2014).

For testing the possible contribution of Als3 to enolase localization at the surface of fungal cells, *S. cerevisiae* cells overexpressing Als3 protein were used, and the result was compared with the enolase binding properties of other GPI anchored *C. albicans* proteins (Eap1 and Hwp1) considered as references in the same model (Figure 5). These *C. albicans* adhesins were expressed at the surface of the *S. cerevisiae* surrogate as fusion proteins with a hemagglutinin (HA) tag near the C terminus, followed by the Ser/Thr-rich region of Epa1 from *C. glabrata* and the C-terminal domain of the *S. cerevisiae* protein Cwp2, introduced for efficient anchoring to the cell wall (Nobbs, Vickerman, Jenkinson, 2010). By using anti-HA antibodies, we verified that these proteins had a comparable surface expression level between individual experiments. A control strain expressing the *S. cerevisiae* Cwp1 protein was used to test the possible effect of an additional fragment of the *C. glabrata* protein on adhesion and protein binding. Whilst maintaining a similar level of expression of *C. albicans* adhesive proteins at the surface of *S. cerevisiae*, the highest enolase binding capacity was demonstrated for the Als3 protein, confirming our earlier observations (Table 1), whereas the other *C. albicans* proteins tested—Eap1 and Hwp1—could make a contribution, although significantly lower, to interactions with cytoplasmic enolase at the fungal cell

surface. The low signal obtained for enolase binding to Cwp1 excluded the participation of the other supporter fragments of the construct in these interactions, as well as any significant contribution of the other components of the surrogate *S. cerevisiae* cell surface.

2.5. Characteristics of the enolase-Als3 interaction: binding kinetics and involved adhesin molecule fragments

To verify that Als3 interacts with enolase, as indicated by the affinity chromatography, cross-linking and experiments using *S. cerevisiae* cells exposing selected candidal adhesins, we purified the Als3 protein from surface of *C. albicans* cells using a previously described method (Seweryn *et al.*, 2015). The surface plasmon resonance (SPR) measurements were employed in which the Als3 protein was deposited on the surface of a CM3 chip in accordance with the manufacturer’s protocol to better reflect its surface-bound state and to simplify the model of interaction. As a reference, a second cell on the CM3 chip was activated with the EDC/NHS system and blocked with ethanolamine. Based on the resonance curves obtained during contact of the immobilized Als3 protein with a number of enolase dilutions, the dissociation constant of the formed complex was estimated at 36.7 nM using homologous plots of association and dissociation curves, representing the Langmuir model of interaction (Figure 6). A high binding affinity between these two proteins was thus confirmed.

To address the question of which parts of the Als3 molecule were principally involved in the interaction with enolase, we used *S. cerevisiae* cells expressing the full sequence or deletion mutants of Als3 (Figure 7A). These mutants were used previously to map the regions of Als3 responsible for adhesion to artificial surfaces, and thus essential for biofilm formation, and that participate in the interaction with ECM proteins (Silverman *et al.*, 2010;

Bamford, Nobbs, Barbour, Lamont, Jenkinson, 2015). In the method used in our present study, *S. cerevisiae* cell suspensions were allowed to equilibrate with solutions containing biotinylated enolase at increasing concentrations over the same range as in the experiments described above. The surface exposure of all Als3 variants on *S. cerevisiae* cells was determined in each experiment using antibodies that recognize a C-terminal HA-tag, as described previously (Nobbs *et al.*, 2010). A comparison of enolase binding to these mutants, presented in Figure 7B, was performed for the same enolase concentration (250 nM), ensuring the saturation of the binding sites. The binding to the full Als3 sequence on the *S. cerevisiae* cells was assigned a value of 100%, whereas that to the yeast cells expressing plasmid alone was taken as 0%. We found that the Ig-like N-terminal region (aa166-225; aa218-285; aa270-305; 277-286) and the central repeat domain (aa434–830) were essential regions of Als3 for the interaction with enolase as the corresponding mutants showed only about 20% binding. In contrast, the elimination of the sequence across the amyloid-forming region of Als3, aa325-331, which is believed to induce adhesin clustering (Beaussart *et al.*, 2012), did not reduce enolase binding.

2.6. The surface-located enolase of *C. albicans* is involved in the regulation of fungal Als3 adhesin interactions with host plasma proteins

The enolase binding to Als3 raised questions regarding the mechanism of its interactions with host proteins on the *Candida* cell surface. The most possible is an Als3-mediated bridging of enolase-host protein complexes on the fungal cells. To test this, we employed *S. cerevisiae* cells expressing surface *C. albicans* Als3 protein deprived of its amyloid sequence, as this variant has the same enolase binding properties as full-length Als3. The use of this deletion mutant overcomes the problem of yeast cell aggregation

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3 during FACS analysis. The cells were allowed to interact for 90 minutes with combinations
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5 of the fluorescein-stained host proteins HK or HPG, and Alexa Fluor 647-stained enolase, at
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7 different concentration ranges. After cell washing, the stained cells were analyzed for the
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9 presence of both types of proteins at their surface to examine for mutual protein
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11 interactions (Figure 8).
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15 At the low concentration of enolase (below 100 nM) in the cell surroundings, an
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17 increased deposition of enolase on the cell surface was observed. And it was not affected
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19 by the presence of any host proteins (Figure 8A, B). This concentration range of enolase
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21 corresponds to its relative binding constant (36.4 nM), estimated as the concentration of
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23 enolase that will achieve a half-saturation of binding sites on the *S. cerevisiae* cells (3×10^7
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25 cells) harboring surface Als3 molecules (Figure 5). It was consistent also with the value
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27 determined in our SPR experiment using immobilized Als3 protein (36.7 nM, Figure 6).
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29 However, with a further increase in the enolase concentration (above 100 nM), we
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31 observed a lowering of its surface binding in the presence of both host proteins. Moreover,
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33 the enolase binding to the Als-3 exposing cells was more sensitive to kininogen than to
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35 plasminogen presence, at the same concentration range of host proteins. These observed
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37 effects may have been the result of the possible formation of the soluble complexes
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39 between enolase and a specific host protein that lowered the level of enolase available for
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41 Als3 saturation at the yeast cell surface.
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50 Another explanation for these observed results assumes that enolase and host proteins
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52 can compete for a binding site on the Als3 molecule. A possible interaction between Als3
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54 and HK was identified in our previous study (Seweryn *et al.*, 2015), but the participation of
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56 Als3 in HPG binding by *C. albicans* has never been proposed. Hence, we verified an HPG
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58 interaction with Als3 and compared its binding affinity with that determined for HK using
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3 biotinylated plasma proteins contacting pBC-Als3 yeast cells (Figure 8C). This comparative
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5 analysis revealed that both proteins could bind to surface-exposed Als3 but with different
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7 binding strengths as a greater affinity of Als3 was detected for HK compared with HPG. To
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9 map the Als3 regions involved in the interaction with both host proteins, we again used cells
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11 expressing various deletion mutants of Als3 on their surface (Figure 8D). We concluded that
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13 similar regions of the Als3 molecule are essential for the binding to both host proteins.
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15 However, the interaction with HK was more sensitive to the elimination of any part of the
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17 Als3 molecule than that with HPG, suggesting tighter Als3 binding to HK than to HPG. This
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19 was also reflected by the above-demonstrated saturation of binding to *S. cerevisiae* pBC-
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21 Als3.
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28 We finally evaluated the host protein interactions with the yeast cells in the presence
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30 of an increased concentration of enolase. The results, presented in Figures 8E and 8F,
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32 confirmed the possible competition of host proteins and enolase for the same binding site.
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34 Moreover, the competitive potential of enolase towards HPG was higher compared with
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36 HK. These findings shed new light on the possible role of enolase at the *C. albicans* cell
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38 surface and highlight for the first time that the regulatory properties of enolase include the
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40 surface adsorption of host proteins essential for fungal infection.
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46 3. Discussion

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48 The multiplicity and diversity of *a priori* non-characteristic functions mediated at the
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50 surface of various cells by “moonlighting” proteins have led to the search for conditions that
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52 determine their unexpected location and interactions, i.e. that underlie their new roles.
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54 Enolase is one of the most common of these proteins and is found at the surfaces of almost
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56 all types of cells i.e. from bacteria (Pancholi & Fischetti, 1998; Derbise, Song, Parikh,
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3 Fischetti, Pancholi, 2004), fungi (Eroles *et al.*, 1997) and parasites (Bhowmick, Kumar,
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5 Sharma, Coppens, Jarori, 2009) to human immune (Wygrecka *et al.*, 2009), cancer
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7 (Didiasova, Wujak, Wygrecka, Zakrzewicz, 2014; Didiasova *et al.*, 2015) and neuronal
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9 (Haque, Polcyn, Matzelle, Banik, 2018) cells.
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13 The location and role of the multifunctional enolase protein at the fungal cell surface
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15 remain enigmatic. The principal question thus concerns the conditions that favor enolase
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17 deposition on the yeast cells. As the scientific literature currently provides incomplete
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19 information on this topic, we first determined the cell growth phase and preferred medium
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21 for *C. albicans* ATCC10231 strain propagation that would result in the maximal accumulation
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23 of enolase on the cell surface. We documented that the long-lasting stationary phase of the
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25 cell growth promoted enolase deposition on the yeast-like *C. albicans* cells. For further
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27 comparison, we used several different growth media that simulated the conditions found
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29 in natural niches settled by *C. albicans* and did not find significant differences in the enolase
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31 surface content compared with cells growing in YPD media, although some variation in this
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33 regard was observed for non-albicans *Candida* species (Karkowska-Kuleta *et al.*, 2019).
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41 Silva and co-workers (Silva *et al.*, 2014) had found in their prior study that enolase was
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43 present in the matrix of mono-species fungal biofilms. However, *C. albicans* also forms
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45 mixed **biofilms** with bacteria, such as anaerobic *P. gingivalis*, that is well protected in aerobic
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47 **conditions** by fungal cells (Bartnicka *et al.*, 2019). We noted increased surface **display** of
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49 enolase under anoxia and in aerobic cell growth in the presence of bacterial cells compared
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51 with a mono-species fungal biofilm (Bartnicka *et al.*, 2019). There are a plethora of factors
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53 that might be responsible for this phenomenon. First of all, there are the quorum sensing
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55 molecules (QSM) produced by the action of both types of pathogens to influence their own
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57 or their partner's propagation, including fungal farnesol and farnesoic acid, as well as
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3 bacterial autoinducer-2. There were no significant changes however in the surface enolase
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5 levels on the cells treated with both types of QSM at the concentration range identified
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7 under mixed biofilm conditions. Surprisingly also, we found in our present analyses that
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9 bacterial LPS did not influence the enolase level on the fungal cell surface. However, in the
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11 case of human cells, it was shown previously that LPS can trigger the translocation of host
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13 enolase to the extracellular space due to fluctuations in the intracellular calcium level
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15 (Didiasova *et al.*, 2015) or post-translational modifications (Zakrzewicz *et al.*, 2018). Another
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17 stimulating factor that may potentially influence the surface deposition of fungal enolase is
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19 the proteolytic activity of bacteria. Indeed, a previous comparison of mixed biofilms formed
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21 by fungi with the wild type *P. gingivalis* W83 strain or its mutant deprived of the leading
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23 group of cysteine proteases, the gingipains, revealed a two-fold lower level of surface fungal
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25 enolase during contact with the mutant strain, but still at a higher level than in mono-
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27 species fungal biofilms formed under the same conditions (Bartnicka *et al.*, 2019).
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35 The increasing bacterial proteolytic activities resulted in a gradual loss of fungal cell
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37 integrity. This may point to enolase deposition after its outflow from damaged cells. Notably
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39 however, Silva and co-workers (Silva *et al.*, 2014) demonstrated that the enolase from lysed
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41 *C. albicans* cells of strain 5373 was not stable outside the cells. On the other hand, the
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43 isolation of intact protein from the biofilm structure formed by the cells of strain 3232
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45 suggested that the quick binding of enolase to the outer fungal cell surface or biofilm matrix
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47 can protect the protein from further degradation.
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52 There has only been one publication to date (Angiolella *et al.*, 1996) showing that
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54 enolase is associated with glucans in the cell wall. However, to identify the surface attached
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56 proteins, the authors of that study used a β -1,3-glucanase (zymolyase) in the extraction
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58 method. This enzyme also contains mannanase activity that may partially deglycosylate
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some of the surface proteins that could play a receptor function for enolase (Beucher *et al.*, 2009). Moreover, these authors did not analyze high molecular mass proteins or identify any complexes.

Before looking for an enolase receptor at the *C. albicans* cell surface in our present analysis, we first tested the possibility that enolase rebinds to the cell surface, as its transport not only to the cell surface but also into the medium has been commonly observed (Sundstrom & Aliaga, 1994; Eroles *et al.*, 1997). As expected, our analysis revealed that the filamentous form of the *C. albicans* fungal cells bound a higher amount of enolase at the cell surface compared with the yeast-like cells.

We searched for a possible receptor capable of enolase binding using two independent approaches, and we found that Als3—the GPI anchored glycoprotein—and some cellular enzymes were putative enolase binding proteins. Among them, glucose-6-phosphate isomerase, 6-phosphogluconate dehydrogenase, and phosphoglycerate mutase belong, like enolase, to the “moonlighting” protein group. Besides, it was documented that mouse muscle enolase binds with a high affinity to some glycolytic enzymes, like phosphoglycerate mutase or pyruvate kinase (Merkulova *et al.*, 1997), suggesting that these enzymes can be transferred or located in the form of complexes at the fungal cell surface, possibly stabilizing their structural integrity. Taking into account that these enzymes are rather responsible for translocation or stability of extracellular enolase, in the further analysis we focused on Als3 protein as a putative receptor. In addition, among the typical cell wall proteins, chitinase 2 was also identified (Dünkler, Walther, Specht, Wendland, 2005) but its contribution to enolase binding was by far the lowest.

We verified the participation of Als3 in enolase attachment by testing enolase binding to *S. cerevisiae* cells that expressed different *C. albicans* surface adhesins, including Als3,

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3 Eap1, and Hwp1. These experiments revealed that only Als3 was able to tightly interact with
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5 enolase. We used the popular *S. cerevisiae* model cell system to overexpress the *C. albicans*
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7 *ALS3* gene instead of *C. albicans* deletion mutants, to avoid possible compensatory changes
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9 in the expression of other *ALS* genes, but still keeping in mind the possibility of altered
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11 glycosylation patterns from this exogenous expression (Cota & Hoyer, 2015).
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16 The hypothesis that Als3 is the major Als receptor for enolase is supported by the
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18 findings that the gene encoding this protein has a higher transcriptional level in all types of
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20 *C. albicans* strains, independent of their growth milieu. Moreover, under conditions that
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22 promote hyphal growth, anti-Als3 immunostaining pointed to peak Als3 expression in cells
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24 adopting this morphology (Coleman *et al.*, 2009), with different location preferences for
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26 other members of this protein family. The Als3 protein is linked to β -1,6-glucan (Kapteyn *et al.*,
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28 2000), which explains the previously observed release of enolase from the fungal cell
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30 surface after enzymatic treatment with zymolyase.
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35 Als3 possesses an extensive list of divergent, structurally unrelated binding partners
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37 among host proteins as well as bacterial proteins such as SspB of *Streptococcus gordonii*
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39 (Silverman *et al.*, 2010) and InlJ or Rgp of *P. gingivalis* (Sztukowska *et al.*, 2018; Bartnicka *et al.*,
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41 2019). The collective evidence to date suggests that the principal binding regions within
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43 the Als3 molecule are localized within its N-terminal domain (NT), which possesses broad
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45 peptide recognition properties (Salgado *et al.*, 2011) and was found to be important for
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47 interactions with host proteins such as the E- or N-cadherins (Phan *et al.*, 2007), collagen,
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49 fibrinogen or fibronectin, as well as the *S. gordonii* SspB protein (Bamford *et al.*, 2015). On
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51 the other hand, the amyloid-forming region (AFR), localized at the very end of the NT
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53 domain of Als3, and found to be critical for the *C. albicans* cell aggregations (Lin *et al.*, 2014),
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55 is not crucial for interactions with bacterial SspB and its deletion only partially affected the
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biofilm formation by Als3-exposed *S. cerevisiae* cells on silicone surfaces coated with ECM proteins. The central repeat domain (CRD) of Als3 was found not to be required for bacterial adhesion, but its deletion affected the interaction with ECM proteins, indicating a possible role of this domain in the folding of the N-terminal region of Als3 or the exposition of additional adhesive areas in the Als3 molecule (Bamford *et al.*, 2015).

A similar strategy in identification of Als3 fragments important for the interaction with *C. albicans* enolase showed that this protein preferred to interact with the same regions of the Als3 molecule as ECM, without any binding to the AFR region and with stronger preference to the CRD fragment. Comparing all these results, it could be concluded that Als3 molecule interacts relatively easily with proteins of various characteristics, presenting broad substrate specificity. The immobilization of purified Als3 on the chip surface enabled the determination of its enolase binding properties, characterized by the dissociation constant of 36.7 nM. This is the first information about the possible mechanism of enolase display at the fungal cell surface, with involvement of typical fungal adhesins, and a possible explanation of enolase surface location.

The main role of enolase exposed at pathogen cell surfaces has been attributed to the binding of host plasminogen, which can facilitate pathogen migration through the host tissues and consequently the progression of infection (Irigoyen, Muñoz-Cánoves, Montero, Koziczak, Nagamine, 1999; Crowe *et al.*, 2003). Plasminogen in the host plasma is converted to plasmin, which further degrades fibrin but can also digest extracellular matrix proteins and activate collagenases. Plasminogen bound to fungal cells can also be activated to plasmin by its treatment with specific, externally added activators i.e. tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Irigoyen *et al.*, 1999; Jong *et al.*, 2003).

The possible role of enolase as “a bridging molecule” between the fungal adhesin Als3 and host plasminogen is an attractive hypothesis, but its actual functioning seems to be much more intricate. Using enolase and plasminogen labeled with different fluorescent dyes and yeast cells overexpressing *C. albicans* Als3, we here identified, for the first time, how such interactions can be executed. We first observed that an increased host protein concentration caused a decrease in enolase binding to Als3-exposed *S. cerevisiae* cells. On the other hand, the binding of host proteins in this system was also observed, suggesting a potential interaction between Als3 and host proteins. For the results identified for plasminogen, we used kininogen (HK) as a sort of reference host protein, the binding of which on *Candida* spp. cells was well documented (Karkowska-Kuleta, Kozik, Rapala-Kozik, 2010; Karkowska-Kuleta *et al.*, 2011). HK binding by fungal enolase, as well as by Als3, has been previously observed and characterized by dissociation constants (K_D) in the same range for both fungal proteins (ca. 200 nM). These results confirmed the possible competition of HK and enolase for the binding site on Als3 molecule. Similar results were obtained for plasminogen in our present analysis, indicating that Als3 can also function as a receptor for this host protein. Plasminogen binding to Als3 was previously reported only for *C. parapsilosis* homologs of Als adhesin, with the reported kinetic parameters in a comparable range (Karkowska-Kuleta *et al.*, 2017).

It is noteworthy that the identification of Als3 in cell wall protein (CWP) extracts is quite difficult and depends on the extraction method used, electrophoresis conditions, as well as the protein staining procedures. This is likely to explain why this protein has rarely been identified in prior mass spectrometry analysis of *Candida* cell wall proteins that may participate in host protein binding. To further verify this observed interaction, we mapped the regions of Als3 involved in the interaction with HPG and HK and found that the same

domains of this protein required for enolase binding were also essential for interactions with these host proteins. This supports the possibility of a competition between enolase and host proteins for binding to Als3. Moreover, our current observation that HK has a higher binding affinity for Als3 than HPG in the *S. cerevisiae* model was consistent with our finding of the stronger competition between enolase and HK compared with the enolase-HPG pair in the binding to Als3.

The involvement of the surface Als3 protein and enolase, both at the fungal cell surface, as well as those found in solution, may play an important role in the subtle modulation of discussed processes by *C. albicans* and thereby tip the balance in favor of the pathogen. Hence, the interactions of these fungal and human proteins and their further impact on the development of infection is multifaceted and requires further detailed research.

4. Experimental procedures

4.1. Fungal strains and growth conditions

Candida albicans strain ATCC® 10231™ (3147) was purchased from American Type Culture Collection (Manassas, VA). *Saccharomyces cerevisiae* strains used in this study are listed in Table 2. *C. albicans* cells were routinely cultured with shaking (170 rpm) in a liquid YPD medium (1% yeast extract, 2% soybean peptone and 2% glucose; Sigma, St. Louis, MO) at 30°C for 16 hours. *S. cerevisiae* strains expressing *C. albicans* adhesins were cultured without uracil at 30°C for 48 hours in a liquid complete synthetic medium (CSM) (Formedium, Norfolk, United Kingdom) supplemented with 0.67% yeast nitrogen base and 2% glucose (Sigma), whereas for cultures of the parent strain *S. cerevisiae* BY4742, a supplementation with 25 µg/mL uracil was applied under the same conditions (Beaussart *et al.*, 2012; Bamford *et al.*, 2015). Cell numbers for both species were determined by optical

density measurements at 600 nm after harvesting the cells by centrifugation (3000 *g*, 5 minutes) and washing three times with 1 mL of PBS buffer, pH 7.4. The appropriate growth medium dedicated to particular yeast species with the addition of 1.5% agar was used to store the fungal cultures.

4.2. Determination of *C. albicans* growth curve and cell viability

Samples of *C. albicans* yeast cells (10^7 cells/mL) were placed into MaxiSorp 96-well microtiter plates (Nunc, Roskilde, Denmark) in 200 μ L of YPD medium per well and then incubated under cover at 30°C with shaking in a Synergy™ H1 microplate reader (BioTek Instruments, Winooski, VT) for 18 hours. The OD at 600 nm was measured every hour. Additionally, to test the cell viability, aliquots containing the same number of cells were plated into the wells at 2-hour intervals and incubated in YPD medium at 30°C. After 19 hours of incubation, the cells were washed and incubated with tetrazolium dye (XTT) (Thermo Fisher Scientific, Waltham, MA) for 2 hours at 30°C, and the absorbance was measured at 450 nm.

4.3. Immunodetection of surface-located *C. albicans* enolase

C. albicans yeast cells grown were transferred into Eppendorf tubes and washed twice with 1 mL of PBS. Then 100 μ L aliquots of cell suspensions containing 10^7 of yeast-like cells were placed into new tubes. The hyphal forms of *C. albicans* were obtained after a further incubation of the fungal cells (10^6) for 3 hours in 100 μ L of RPMI 1640 medium at 37°C in MaxiSorp microplates (Nunc). Prior to the addition of the antibodies, to avoid their non-specific binding, the surface of the fungal cells, both yeast-like and hyphal forms, as well as the unoccupied microplate well surface were blocked by incubation with 300 μ L of 1% bovine serum albumin (BSA) in PBS for 1 hour at 37°C. Moreover, the planktonic cells were

transferred after the binding of antibodies to new Eppendorf tubes prior addition of the substrate for peroxidase. For detection of enolase at the surface of yeast-like cells growing in 100 μ L of YPD medium in the wells of the microplate, 10^7 cells/mL were fixed with 100 μ L of 3.7% paraformaldehyde for 30 minutes at appropriate time intervals, to stop further cell growth. At each timepoint the yeast cells in the tubes were centrifuged (5 minutes, 3000 g) and washed three times with 100 μ L of PBS, and the cells on the microplate were washed three times with 200 μ L of PBS. The detection of surface-displayed enolase for both morphological forms was performed with the addition of 50 μ L of mouse monoclonal anti-enolase antibodies (1 μ g/mL) in PBS (Invitrogen, Carlsbad, CA) for 1 hour at 37°C, followed by 50 μ L of goat anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP) (1 μ g/mL, Abcam, Cambridge, UK) for 1 hour at 37°C. The enzymatic reaction was developed with 50 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) as the HRP substrate for 3 minutes and stopped with the addition of 50 μ L 2 M HCl. The supernatant was then transferred into a new microplate and the absorbance at 450 nm was measured using a Power Wave X Select microplate reader (BioTek Instruments).

An analogous procedure was used for microscopic observations; however, to obtain *C. albicans* hyphal forms, blastospores from a 16-hours culture were transferred to the wells of a glass bottom 96-well plate (CellVis, Mountain View, CA, USA) coated with poly-L-lysine (10^6 cells per well) and incubated in RPMI 1640 for 3 hours at 37°C. The next steps were common to both morphological forms. After washing with PBS, cells were incubated with 1% BSA, then with 50 μ L of mouse monoclonal anti-enolase antibodies (1 μ g/mL, Invitrogen) in PBS at 37°C for 1 hour and, subsequently, with Alexa Fluor 488 fluorescently labeled donkey anti-mouse secondary antibodies (1 μ g/mL, Abcam, Cambridge, UK) in PBS for 1 hour. The unbound antibodies were washed away with PBS. Blastospores were transferred

from Eppendorf tubes into the wells of a 96-well plate (CellVis) and centrifuged. Imaging was performed using an Olympus IX73 microscope (Olympus, Tokyo, Japan) equipped with a Hamamatsu Orca Spark camera (Hamamatsu, Hamamatsu City, Japan) and a UPLXAPO60XO lens (Olympus). Image analysis was performed using CellSense software (Olympus). To avoid changes in fluorescence intensity due to image acquisition parameters, all images were captured with the same integration times, excitation light intensity, and camera sensitivity. The fluorescence intensity for individual cells (blastospores and filamentous forms) was analyzed (Allikalt, Laasfeld, Ilisson, Kopanchuk, Rinken, 2020). The obtained mean fluorescence intensity (the total fluorescence intensity of the selected area divided by the cell area) determined by a method proposed by Hosseinzadeh and Urban (Hosseinzadeh & Urban, 2013) depends only on the amount of bound fluorophore. The cell borders were annotated manually as one pixel wide lines, in pictures taken in a bright field (Supplementary file – Figure S4). Only the hyphae or their fragments directly in the plane of focus were analyzed.

4.4. Identification of *C. albicans* surface enolase by tryptic shaving

After the growth of *C. albicans* cells in YPD medium at 30°C for 16 hours, aliquots containing 5×10^8 cells were transferred to 20 mL of different growth media and further cultured aerobically for 16 hours or anaerobically for 72 hours at 37 °C (30°C only for the control culture in YPD) on an orbital rotary shaker MaxQ 6000 (170 rpm) (Thermo Fisher Scientific). Anoxic conditions were obtained using a GENbox jar with a GENboxanaer generator (bioMérieux SA, Marcy l'Etoile, France). The following media were used for subsequent cultures: (i) AS – artificial saliva comprising 2.5 g/L mucin from a porcine stomach, type III, 10.0 g/L animal peptone, 5.0 g/L trypticase peptone, 5.0 g/L yeast extract,

5 mg/L hemin, 1 mg/L menadione, 60 mg/L urea (Sigma), 174 mg/L arginine (BioShop) and 2.5 g/L KCl, pH 7.0; (ii) AU – artificial urine composed of 0.65 g/L CaCl_2 , 0.65 g/L MgCl_2 , 4.6 g/L NaCl, 2.3 g/L Na_2SO_4 , 0.65 g/L $\text{Na}_3\text{C}_3\text{H}_5\text{O}(\text{CO}_2)_3$, 0.02 g/L $\text{Na}_2\text{C}_2\text{O}_4$, 2.8 g/L KH_2PO_4 , 1.6 g/L KCl, 1.0 g/L NH_4Cl (Avantor Performance Materials Poland S.A), 25 g/L urea, 1.1 g/L of creatinine (Sigma) and 0.3% glucose, pH 5.8; (iii) SDL – Sabouraud dextrose liquid medium – dextrose 20.0 g/L, casein 5 g/L, peptic digest of animal tissue 5 g/L, pH 5.6; (iv) YPD – 1% yeast extract, 2% soybean peptone and 2% glucose, pH 6.0 (when cultured under anaerobic conditions in this medium - AN). After subsequent harvesting of 5×10^9 cells by centrifugation (5 minutes, 3000 g), cell surface shaving with trypsin was performed, followed by the further identification of proteins based on the identification of released peptides by LC-MS/MS using a Dionex Ultimate 3000 UHPLC system coupled to an HCTUltra ETDII mass spectrometer, as described previously in detail (Karkowska-Kuleta *et al.*, 2019). The relative percentage of enolase was calculated after the NSAFs (normalized spectral abundance factors) were determined for each protein identified in the mixture (the spectral counts (SpC) of a protein divided by its length (L); this value is then divided by the sum of all SpC/L) (Zybailov *et al.*, 2005; Zybailov *et al.*, 2007).

4.5. Analysis of the impact of fungal cell environment on the surface localization of *C.*

***albicans* enolase**

C. albicans cells were grown in YPD for 16 hours at 30°C with the addition of 100 ng/mL lipopolysaccharide (LPS; Sigma), 1 mM hydrogen peroxide (Sigma), 200 μM farnesol (F) (trans,trans-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol; Sigma); 200 μM farnesoic acid (FA) (Echelon Biosciences Inc, Salt Lake City, UT), or 2 μM autoinducer-2 (AI-2) ((S)-4,5-dihydroxy-2,3-pentanedione; Biosynth, Staad, Switzerland). Cells grown only in YPD

medium served as a control. After culturing, the fungal cells (2×10^7) were centrifuged (3000 g, 5 minutes) and washed three times with PBS buffer. Then, the immunodetection of surface-exposed enolase was performed as described above with the use Alexa Fluor 488 conjugated secondary antibodies and subsequently the flow cytometry analysis measurements were carried out on a BD LSRFortessa™ cell analyzer (Franklin Lakes, NJ) with an excitation wavelength of 488 nm and an emission wavelength of 519 nm. The values obtained for cells incubated only with secondary antibodies were subtracted from the values obtained for the tested samples.

4.6. Isolation of *C. albicans* cell surface-exposed proteins and purification of Als3 protein

Fungal cell wall-associated proteins were isolated as previously described (Karkowska-Kuleta *et al.*, 2017) with 2 U of β -1,6-glucanase (Takara Bio Inc.) per 0.4 g of *C. albicans* cells (wet weight) that had been grown in filamentous form for 72 hours in RPMI 1640 medium at 37°C. After incubation of hyphae with the enzyme in 1 mL of McIlvaine buffer for 24 hours at 37°C with gentle shaking, the integrity of the cell membranes was confirmed by Trypan Blue (Sigma) staining and the supernatant containing isolated proteins was collected and dialyzed against PBS buffer for 48 hours at 4°C. Als3 protein was purified from the obtained protein mixture with the use of ion-exchange chromatography on a Resource™ Q column (GE Healthcare, Uppsala, Sweden) followed by gel filtration on a Superdex 200 HR column (GE Healthcare/Amersham Biosciences, Little Chalfont, UK) in accordance with a previously described method (Seweryn *et al.*, 2015). Protein concentrations were determined using the Bradford method (Bradford, 1976). The quality and purity of the obtained preparations were verified by SDS-PAGE electrophoresis in the Laemmli system, and the accuracy of

protein purification was assessed using LC-MS/MS, as described earlier (Seweryn *et al.*, 2015).

4.7. Purification of cytoplasmic enolase

C. albicans cytoplasmic enolase was purified as previously described (Seweryn *et al.*, 2015) in accordance with the method of Ballantyne and Warmington, 2000, with minor modifications (Ballantyne & Warmington, 2000). Briefly, *C. albicans* cells grown in YPD medium at 30°C were washed twice with PBS buffer and then subjected to cell breakage in ice-cold water with protease inhibitors (Complete EDTA-free, Roche, Penzberg, Germany) by sonication (10 sets of 1 minute, interval 0.8) with a Sonic Ruptor 400 Ultrasonic Homogenizer (Omni International, Kennesaw GA). After removing the damaged cells by centrifugation (21000 *g*, 30 minutes), solid ammonium sulphate was added to the supernatant to a final saturation of 65%, and then to 100% after another centrifugation step and discarding the precipitant. A protein precipitate obtained at this stage was dissolved and dialyzed against 20 mM Bis-Tris buffer, pH 6.4 and applied to a Mono Q column (Pharmacia Biotech, Piscataway, NJ) and then to a MonoS HR 5/5 column (Pharmacia Biotech) after dialysis of enolase-containing fractions to 10 mM sodium acetate buffer, pH 4.8. Elution for both chromatographic steps was performed in the appropriate buffer using an 80 mL gradient of 0–1 M NaCl at a flow rate of 1 mL/min. *C. albicans* enolase was identified in the collected eluates using SDS-PAGE and protein identification with LC-MS/MS, as described earlier (Seweryn *et al.*, 2015).

4.8. Binding of biotinylated enolase to the fungal cell surface

C. albicans was cultured in liquid YPD medium for 18 hours at 30°C, whereas *S. cerevisiae* wild and mutant strains were cultured in liquid CSM medium for 48 hours at 30°C. Optical

densities were then measured at 600 nm. Culture aliquots were transferred to Eppendorf tubes (1×10^7 *C. albicans* cells and 3×10^7 *S. cerevisiae* cells) or, in the case of *C. albicans* only, to the wells of a Maxisorp microplate (1×10^6) and additionally grown in RPMI 1640 medium at 37°C for 3 hours to obtain filamentous forms. After each step, cells were washed three times with 200 μ L of PBS buffer, pH 7.4, and cells in suspension were centrifuged each time at 3000 *g* for 3 minutes. To avoid any non-specific binding the unoccupied surface in the wells of the microplate was blocked with 300 μ L of 1% BSA for 1 hour at 37°C and the cells in the Eppendorf tubes were transferred to new tubes just prior to the detection of bound protein. 50 μ L of *C. albicans* enolase (Eno-Bt), biotinylated using biotin N-hydroxysuccinimide ester (Sigma) according to a previously described procedure (Rapala-Kozik *et al.*, 2008), was added to the cells at a concentration range of 25-200 nM for *C. albicans* and 6.25-75 nM or only 250 nM for *S. cerevisiae*, and incubated for 1 hour at 37°C. After washing out unbound protein, 50 μ L of horseradish peroxidase-conjugated streptavidin (SA-HRP, MP Biomedicals) diluted 1:4000 in PBS was incubated with fungal cells for 1 hour at room temperature in the dark. The amount of bound Eno-Bt was measured with the addition to the cells of 50 μ L of substrate for HRP, TMB (Sigma) and the reaction was stopped after 2 minutes with 50 μ L of 2 M HCl. After centrifugation, 90 μ L of the supernatant was transferred into wells of a standard 96-well microtiter plate (Sarsted, Germany) and the absorbance at 450 nm was measured in a Power Wave X Select microplate reader (BioTek Instruments). The control readings were the values calculated for the yeast-like or hyphal forms incubated without enolase. To compare the relative fusion protein expression level at the surface of *S. cerevisiae* mutant strains, 3×10^7 yeast cells were transferred to Eppendorf tubes and incubated with 50 μ L of a 1 μ g/mL solution of mouse anti-HA antibody (ZYMED Laboratories) in PBS for 1 hour at 37°C, washed to remove

unbound primary antibodies, and further incubated with 50 μ L of secondary anti-mouse antibodies (1 μ g/mL) conjugated with HRP. The cells were then transferred to new tubes, and the reaction was developed with 50 μ L TMB for 40 sec, stopped with 50 μ L of 2 M HCl, and after cell removal by centrifugation (3000 *g*, 5 minutes), 90 μ L of supernatant was transferred to the microplate, and the absorbance was measured as described above.

A similar procedure was used for microscopic observations, however, enolase was labelled with NHS-fluorescein (1 mg per 100 μ L of dimethylformamide; Sigma) in 0.1 M carbonate buffer at pH 8.3 at 4°C for 2 hours and then dialyzed against PBS, pH 7.4 at 4°C for 48 hours. *C. albicans* hyphal forms, as described above, were grown in the wells of a glass bottom 96-well microplate (CellVis), incubated with enolase for 1 hour at 37°C. Imaging was performed using an Olympus IX73 microscope.

4.9. Identification of *C. albicans* enolase-binding cell wall proteins with the use of affinity chromatography and chemical cross-linking

Affinity chromatography and chemical cross-linking were performed as described previously in detail (Kozik *et al.*, 2015). Briefly, an affinity gel with covalently conjugated enolase was prepared via the immobilization of 100 μ g enolase in 0.1 M HEPES buffer, 80 mM CaCl_2 , pH 7.5 to 1 mL of Affi-Gel 10 (Bio-Rad, Herkules, CA). The *C. albicans* cell wall proteins (250 μ g) isolated with β -1,6-glucanase were incubated at 37°C for 4 hours with 50 μ L of the gel. After then removing unbound proteins by extensive washing with 1 mL of PBS buffer, bound proteins were eluted with 2% SDS at 95°C for 30 minutes. Proteins eluted from a gel without immobilized enolase but incubated with the mixture of *C. albicans* proteins were used as a control for “non-specific” binding. Additionally, the chemical cross-linking of the biotinylated enolase (10 μ g in 100 μ L PBS, pH 7.4) and β -1,6-glucanase-

extractable *C. albicans* cell wall proteins (250 µg) was performed using 0.5 mM sulfosuccinimidyl 2-([4,4'-azipentanamido]ethyl)-1,3'-dithiopropionate (sulfo-SDAD) (Thermo Fisher Scientific). Covalently linked pairs of biotinylated enolase and other fungal cell wall proteins were adsorbed on MagnaBind streptavidin beads (Thermo Fisher Scientific) and released after washing out unbound proteins by treatment with 2.5% β-mercaptoethanol and 2% SDS for 30 minutes. Proteins eluted after incubation of streptavidin beads alone with non-biotinylated proteins were used as a “non-specific” binding control. Proteins eluted after affinity chromatography were separated by SDS-PAGE and bands that were not present in the control samples were excised and identified using LC-MS/MS as described above.

4.10. Characterization of the Als3 interaction with *C. albicans* enolase using SPR measurements

The kinetic and thermodynamic analysis of *C. albicans* Als3 binding to enolase was performed using a BIACORE 3000 system (GE Healthcare, Uppsala, Sweden). After dialysis in 10 mM HEPES buffer, pH 7.4, Als3 was immobilized onto a CM3 sensor chip surface (GE Healthcare), using the Amine Coupling Kit (GE Healthcare). After activation of the chip surface with 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 200 mM N-hydroxysuccinimide (NHS), Als3 in 10 mM sodium acetate buffer (pH 4.0) was injected over the chip surface at a flow rate of 10 µL/min. The obtained immobilization level reached 90 resonance units (RU). The running buffer - 10 mM HEPES with 150 mM NaCl and 0.005% surfactant P20 (pH 7.4) - and the solutions of enolase (in the concentration range of 5-100 nM) in the same buffer were injected at a flow rate of 30 µL/min at 25°C. The association and dissociation time was 180 seconds. The injection of 1 M NaCl at a flow rate of 30 µL/min

for 30 seconds was used as a regeneration step. The obtained results were analyzed using BIAevaluation 4.1 software (GE Healthcare). The equilibrium dissociation constant (K_D) was calculated by fitting a homologous model that separately determines the dissociation and association rate constants (k_d and k_a).

4.11. Analysis of the *C. albicans* cell surface interaction with fungal enolase and human proteins

Flow cytometry measurements were carried out on a BD LSRFortessa™ cell analyzer (Franklin Lakes, NJ) and with the *S. cerevisiae* strain UB2316. Aliquots of 5×10^6 yeast cells were transferred into Eppendorf tubes, suspended in 500 μ L PBS buffer, and centrifuged for 3 minutes (3000 *g*). Human proteins—HK and HPG—were labelled with NHS-fluorescein (1 mg per 100 μ L of dimethylformamide; Sigma) in 0.1 M carbonate buffer at pH 8.3 at 4°C for 2 hours and enolase with a 30-molar excess of Alexa Fluor 647 (Thermo Fisher Scientific) solution prepared in dimethyl sulfoxide (DMSO) at 37°C for 1 hour. The labelled protein was then dialyzed against PBS, pH 7.4 at 4°C for 48 hours after which the protein concentration was tested using a NanoDrop spectrophotometer. Yeast enolase conjugated with Alexa Fluor 647 was added to cell pellets at increasing concentrations (13.3 nM -1700 nM) together with fluorescein-conjugated HPG or HK at two concentrations (25 nM and 50 nM). Yeast cells with added proteins were incubated for 90 minutes at 37°C and the cells were then washed three times with PBS buffer and 100 μ L of paraformaldehyde was added for 15 minutes. The cells were then transferred to glass tubes for flow cytometric analysis and placed at 4°C. On the following day, the samples were subjected to fluorescence analysis at excitation wavelengths of 488 and 633 nm and emission wavelengths of 519 and 660 nm for fluorescein and Alexa Fluor 647-labels, respectively.

Supplementary Materials: Supplementary Table 1. Mass spectrometry identification of *C. albicans* proteins present at the cell surface under different growth conditions.

Supplementary Table 2. Mass spectrometric parameters for identification of putative enolase-binding *C. albicans* cell wall-associated proteins. Supplementary figures:

Supplementary figure S1. *C. albicans* growth curve (up to 48 h) and the detection of enolase at the surface of the yeast cells. Supplementary figure S2. Detection of surface-located enolase on *C. albicans* cells correlated with morphological changes observed during cell growing at increased temperature (30°C versus 37°C). Supplementary figure S3. Stimulation of enolase surface display in *C. albicans* cells growing in the YPD medium supplemented with selected host proteins. Supplementary figure S4. Comparison of the surface of *C. albicans* cells growing in different morphological forms. Supplementary figure S5. The plot of response at $R_{\text{equilibrium}}$ (R_{eq}) against the enolase concentration (SPR analysis) presenting the saturation of the binding sites of ligand by the analyte.

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Data availability: The main data that supports the findings of this study are available in the article and in the supplementary material of this article. The detailed data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Identification of putative enolase-binding candidal cell wall-associated proteins.

C. albicans cell wall-associated proteins were isolated from germ tubes and hyphal forms with β -1,6-glucanase. Putative enolase-binding proteins were isolated from protein mixtures via affinity chromatography (AC) or identified by chemical cross-linking (CL). The number of plus signs corresponds to the frequency with which the protein was identified by the given method across four biological replicates.

ENTRY (ENTRY NAME)	PROTEIN NAME	AC	CL
Q59L12 (ALS3_CANAL)	Agglutinin-like sequence protein 3	++++	++++
P40953 (CHI2_CANAL)	Chitinase 2	+	
O13287 (6PGD_CANAX)	6-Phosphogluconate dehydrogenase	+	++
P30575 (ENO1_CANAL)	Enolase		++++
P83780 (G6PI_CANAL)	Glucose-6-phosphate isomerase		++++
P82612 (PMGY_CANAL)	Phosphoglycerate mutase		++

Table 2. *S. cerevisiae* strains used in this study.

Strain	Description	Reference
<i>Saccharomyces cerevisiae</i> BY4742 UB2022	<i>MATα leu2Δ0 lys2Δ0 ura3Δ0 his3Δ1</i>	(Brachmann <i>et al.</i> , 1998)
UB2155	BY4742 with pBC542 (9.2 kb; ApR; pMB1 <i>ori</i> ; <i>URA3</i>)	(Nobbs <i>et al.</i> , 2010)
UB2157	BY4742 with pBC542- <i>ALS3lg</i>	(Nobbs <i>et al.</i> , 2010)
UB2158	BY4742 with pBC542- <i>EAP1</i>	(Nobbs <i>et al.</i> , 2010)
UB2159	BY4742 with pBC542- <i>HWP1</i>	(Nobbs <i>et al.</i> , 2010)
UB2161	BY4742 with pBC542- <i>CWP1</i>	(Nobbs <i>et al.</i> , 2010)
UB2307	BY4742 with pBC542- <i>ALS3Δ218-285</i>	(Bamford <i>et al.</i> , 2015)
UB2316	BY4742 with pBC542- <i>ALS3Δ325-331</i>	(Bamford <i>et al.</i> , 2015)
UB2418	BY4742 with pBC542- <i>ALS3Δ166-225</i>	(Bamford <i>et al.</i> , 2015)
UB2419	BY4742 with pBC542- <i>ALS3Δ270-305</i>	(Bamford <i>et al.</i> , 2015)
UB2420	BY4742 with pBC542- <i>ALS3Δ277-286</i>	(Bamford <i>et al.</i> , 2015)
UB2421	BY4742 with pBC542- <i>ALS3Δ434-830</i>	(Bamford <i>et al.</i> , 2015)

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Take Away

- *C. albicans* enolase is exposed on the cell surface in different conditions
- Greater enolase abundance was found at the surface of fungal filamentous forms
- Candidal surface adhesin Als3 is involved in the binding of enolase
- N-terminal and central repeat domains of Als3 are crucial for enolase display
- Surface enolase regulates Als3 interactions with plasminogen and kininogen

For Peer Review

Figure legends

Figure 1. *C. albicans* growth curve and the detection of enolase at the surface of the yeast cells. Aliquots of 2×10^6 *C. albicans* yeast cells were placed into the wells of a 96-well microtiter plate in 200 μ L of YPD medium for 19 hours. The cell growth was followed by OD measurements at 600 nm, whereas cell viability was analyzed with XTT tests at specified time intervals. The presence of enolase at the cell surface was detected using mouse monoclonal anti-enolase antibodies (1 μ g/mL) in PBS and goat anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP). Signals were detected using TMB as a substrate for HRP.

Figure 2. Display of enolase on the surface of *C. albicans* cells growing under different environmental conditions. (A) *C. albicans* cells were cultured aerobically in various media (AU – artificial urine, AS – artificial saliva, SDL – Sabouraud dextrose liquid medium) or cultured anaerobically in YPD medium (AN). Surface-exposed proteins were identified by cell surface shaving with trypsin followed by LC-MS/MS. The relative percentage of enolase was calculated after the mathematical normalization of spectral abundance factors. The sum of the normalized spectral abundance factor values calculated for all proteins identified in a given sample was taken as 100%. Bars represent the mean values of 3 determinations $\pm$ standard deviation (SD). (B) *C. albicans* cells were cultured in YPD medium without (C, control) or with one of the indicated supplements (farnesoic acid (FA) (200 μ M), farnesol (F) (200 μ M), autoinducer-2 (AI-2) (2 μ M), lipopolysaccharide from *Escherichia coli* (LPS) (100 ng/mL) or H_2O_2 (1 mM)) for 16 hours at 30°C. Cells were incubated with mouse anti-enolase antibodies and subsequently with secondary anti-mouse antibodies conjugated with Alexa Fluor 488. After gentle cell washing, flow cytometry analysis was

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performed, with fluorometric detection conducted at an excitation wavelength of 488 nm and emission wavelength 519 nm. The control measurements (the values for cells incubated only with secondary antibodies) were subtracted from the total binding values. Data are plotted as means $\pm$ standard error of the mean. Statistical significance levels were determined using one-way ANOVA with Dunnett's multiple comparisons test and are marked relative to the amount of enolase exposed in the YPD medium; **** p <0.0001.

Figure 3. Differences in the enolase levels at the surface of *C. albicans* cells induced to grow as different morphological forms. *C. albicans* cells were grown for 16 hours in YPD medium at 30°C (blastospores) or in RPMI 1640 for 3 hours at 37°C (filamentous forms). (A, B) The amount of surface-exposed enolase was detected with mouse monoclonal anti-enolase specific antibodies and Alexa Fluor 488 labeled anti-mouse secondary antibodies. The measured parameter was the mean fluorescence intensity, which was determined as the ratio of the fluorescence intensity presented by the single cell to its surface area; (C) blastospores (10^7 cells) and filamentous forms (obtained from 10^6 yeast-like cells incubated in wells of MaxiSorp microplates) of *C. albicans* were incubated with mouse monoclonal anti-enolase antibodies and anti-mouse antibodies conjugated with HRP, and the detection was performed using TMB as a HRP substrate with the absorbance measurement at 450 nm. The control readings (the values calculated for the yeast-like or filamentous forms incubated only with HRP-conjugated secondary antibodies) were subtracted from the total binding values. Data are plotted as means from three determinations $\pm$ standard error of the mean. Statistical significance levels were determined using a Student t-test and are marked relative to each other with ** for p <0.01.

Figure 4. Binding of enolase to the surface of the yeast-like and filamentous forms of *C.*

***albicans* cells.** (A) Blastospores (10^7 cells) and the filamentous forms (obtained from 10^6 yeast-like cells incubated for 3 hours at 37°C in 100 μL RPMI 1640 medium in wells of MaxiSorp microplates) of *C. albicans* were incubated with 50 μL biotin-labeled purified cytosolic *C. albicans* enolase (25-200 nM) for 1 hour at 37°C in PBS buffer, pH 7.4. The bound protein levels were determined using the SA-HRP/TMB detection system and the absorbance was measured at 450 nm. The control readings (the values calculated for the yeast-like or hyphal forms incubated without enolase) were subtracted from the total binding values. Data are plotted as means from three determinations $\pm$ standard error of the mean. Statistical significance levels were determined using a Student t-test and are marked with * for $p < 0.05$ and ** for $p < 0.01$. (B) *C. albicans* cells were grown in RPMI 1640 for 3 hours at 37°C to obtain hyphal forms and then were incubated with 50 μL fluorescein-labeled purified cytosolic *C. albicans* enolase for 1 hour at 37°C in PBS buffer, pH 7.4.

Figure 5. Binding of *C. albicans* enolase to *S. cerevisiae* cells harboring surface-expressed

candidal adhesins. *S. cerevisiae* cells (3×10^7) expressing the *C. albicans* adhesins Als3, Eap1, and Hwp1, a control protein Cwp1, and pBC542 vector alone, were incubated with 50 μL of *C. albicans* biotin-labeled enolase in PBS buffer, pH 7.4 for 1.5 h at 37°C with gentle stirring. The amount of bound protein was determined using the SA-HRP/TMB detection system, and the absorbance was measured at 450 nm. The values for the control strain harboring the pBC542 vector alone were subtracted from the other values. The experiment was performed in triplicate and cumulative results presented with data points reported as the means $\pm$ SEM. Binding curves representing data fits with nonlinear regression were generated with GraphPad Prism software.

Figure 6. SPR analysis of the Als3-enolase interaction. Sensograms were obtained after injection of enolase at a concentration range of 5–100 nM and at a flow rate of 30 μ L/min over a CM3 chip containing Als3 immobilized to a level of 90 RU. The binding parameters for the interactions between these fungal proteins were determined after fitting the data illustrated by a black line and are presented with the standard error (SE). The graph illustrating the equilibrium responses (Req) as a function of enolase concentration (up to 400 nM) is presented in the Supplementary materials (Figure S5).

Figure 7. Mapping of the Als3 regions involved in enolase binding. (A) schematic representation of Als3 expressed on the surface of *S. cerevisiae* and corresponding deletion mutants, based on (Bamford *et al.*, 2015), AFR – amyloid-forming region, GPI – glycosylphosphatidylinositol, HA – hemagglutinin; (B) *S. cerevisiae* cells (3×10^7 cells) expressing *C. albicans* adhesin Als3, specific deletion mutants of Als3, or the pBC542 vector alone, were incubated with *C. albicans* biotin-labeled enolase. The amount of bound protein was determined with the SA-HRP/TMB detection system. The values obtained with the vector alone control were subtracted from all other values. The experiment was performed in triplicate and a representative result is shown as the mean $\pm$ standard deviation (SD). Statistical significance levels were determined using one-way ANOVA with a Tukey’s multiple comparisons test relative to pBC-Als3; ** $p < 0.01$, *** $p < 0.001$.

Figure 8. Characteristics of the mutual interactions between the HK and HPG host proteins and Als3 and Eno1 on the yeast cell surface. (A-B, E-F) *S. cerevisiae* cells (5×10^6 cells per point) expressing surface Als3 lacking its amyloid fragment were incubated in suspension (50 μ L) with HPG or HK host proteins conjugated with fluorescein and over an increasing concentration range of enolase (Eno1) conjugated with Alexa Fluor 647. After 90

minutes of incubation and gentle cell washing, flow cytometry analysis was performed, with fluorometric detection conducted at excitation wavelengths of 488 nm or 633 nm and emission wavelengths 519 nm or 660 nm for fluorescein or Alexa Fluor 647, respectively.

(C) The binding of the HK and HPG in the presence of Eno1 was analyzed in the same *S. cerevisiae* model using biotinylated host proteins and the SA-HRP/TMB detection system.

(D) Identification of the Als3 regions important for these mutual interactions was performed using the *S. cerevisiae* surrogate for Als3 as described above and the SA-HRP/TMB detection system.

Figure 1. *C. albicans* growth curve and the detection of enolase at the surface of the yeast cells. Aliquots of 2×10^6 *C. albicans* yeast cells were placed into the wells of a 96-well microtiter plate in 200 μ L of YPD medium for 19 hours. The cell growth was followed by OD measurements at 600 nm, whereas cell viability was analyzed with XTT tests at specified time intervals. The presence of enolase at the cell surface was detected using mouse monoclonal anti-enolase antibodies (1 μ g/mL) in PBS and goat anti-mouse secondary antibodies conjugated with horseradish peroxidase (HRP). Signals were detected using TMB as a substrate for HRP.

110x78mm (300 x 300 DPI)

Figure 2. Exposition of enolase on the surface of *C. albicans* cells growing under different environmental conditions. (A) *C. albicans* cells were cultured aerobically in various media (AU – artificial urine, AS – artificial saliva, SDL – Sabouraud dextrose liquid medium) or cultured anaerobically in YPD medium (AN). Surface-exposed proteins were identified by cell surface shaving with trypsin followed by LC-MS/MS. The relative percentage of enolase was calculated after the mathematical normalization of spectral abundance factors. The sum of the normalized spectral abundance factor values calculated for all proteins identified in a given sample was taken as 100%. Bars represent the mean values of 3 determinations $\pm$ standard deviation (SD). (B) *C. albicans* cells were cultured in YPD medium without (C, control) or with one of the indicated supplements (farnesoic acid (FA) (200 μ M), farnesol (F) (200 μ M), autoinducer-2 (AI-2) (2 μ M), lipopolysaccharide from *Escherichia coli* (LPS) (100 ng/mL) or H₂O₂ (1 mM)) for 16 hours at 30°C. Cells were incubated with mouse anti-enolase antibodies and subsequently with secondary anti-mouse antibodies conjugated with Alexa Fluor 488. After gentle cell washing, flow cytometry analysis was performed, with fluorometric detection conducted at an excitation wavelength of 488 nm and emission wavelength 519 nm. The control measurements (the values for cells incubated only with secondary antibodies) were subtracted from the total binding values. Data are plotted as means $\pm$ standard error of the mean. Statistical significance levels were determined using one-way ANOVA with Dunnett's multiple comparisons test and are marked relative to the amount of enolase exposed in the YPD medium; **** p <0.0001.

168x57mm (300 x 300 DPI)

Figure 3. Differences in the enolase levels at the surface of *C. albicans* cells induced to grow as different morphological forms. *C. albicans* cells were grown for 16 hours in YPD medium at 30°C (blastospores) or in RPMI 1640 for 3 hours at 37°C (filamentous forms). (A, B) The amount of surface-exposed enolase was detected with mouse monoclonal anti-enolase specific antibodies and Alexa Fluor 488 labeled anti-mouse secondary antibodies. The measured parameter was the mean fluorescence intensity, which was determined as the ratio of the fluorescence intensity presented by the single cell to its surface area; (C) blastospores (107 cells) and filamentous forms (obtained from 106 yeast-like cells incubated in wells of MaxiSorp microplates) of *C. albicans* were incubated with mouse monoclonal anti-enolase antibodies and anti-mouse antibodies conjugated with HRP, and the detection was performed using TMB as a HRP substrate with the absorbance measurement at 450 nm. The control readings (the values calculated for the yeast-like or filamentous forms incubated only with HRP-conjugated secondary antibodies) were subtracted from the total binding values. Data are plotted as means from three determinations $\pm$ standard error of the mean. Statistical significance levels were determined using a Student t-test and are marked relative to each other with ** for $p < 0.01$.

168x146mm (300 x 300 DPI)

Figure 4. Binding of enolase to the surface of the yeast-like and filamentous forms of *C. albicans* cells. (A) Blastospores (107 cells) and the filamentous forms (obtained from 106 yeast-like cells incubated for 3 hours at 37°C in 100 μ L RPMI 1640 medium in wells of MaxiSorp microplates) of *C. albicans* were incubated with 50 μ L biotin-labeled purified cytosolic *C. albicans* enolase (25–200 nM) for 1 hour at 37°C in PBS buffer, pH 7.4. The bound protein levels were determined using the SA-HRP/TMB detection system and the absorbance was measured at 450 nm. The control readings (the values calculated for the yeast-like or hyphal forms incubated without enolase) were subtracted from the total binding values. Data are plotted as means from three determinations $\pm$ standard error of the mean. Statistical significance levels were determined using a Student t-test and are marked with * for $p < 0.05$ and ** for $p < 0.01$. (B) *C. albicans* cells were grown in RPMI 1640 for 3 hours at 37°C to obtain hyphal forms and then were incubated with 50 μ L fluorescein-labeled purified cytosolic *C. albicans* enolase for 1 hour at 37°C in PBS buffer, pH 7.4.

168x181mm (300 x 300 DPI)

Figure 5. Binding of *C. albicans* enolase to *S. cerevisiae* cells harboring surface-expressed candidal adhesins. *S. cerevisiae* cells (3×10^7) expressing the *C. albicans* adhesins Als3, Eap1, and Hwp1, a control protein Cwp1, and pBC542 vector alone, were incubated with 50 μ L of *C. albicans* biotin-labeled enolase in PBS buffer, pH 7.4 for 1.5 h at 37°C with gentle stirring. The amount of bound protein was determined using the SA-HRP/TMB detection system, and the absorbance was measured at 450 nm. The values for the control strain harboring the pBC542 vector alone were subtracted from the other values. The experiment was performed in triplicate and cumulative results presented with data points reported as the means $\pm$ SEM. Binding curves representing data fits with nonlinear regression were generated with GraphPad Prism software.

80x55mm (300 x 300 DPI)

Figure 6. SPR analysis of the Als3-enolase interaction. Sensograms were obtained after injection of enolase at a concentration range of 5–100 nM and at a flow rate of 30 $\mu\text{L}/\text{min}$ over a CM3 chip containing Als3 immobilized to a level of 90 RU. The binding parameters for the interactions between these fungal proteins were determined after fitting the data illustrated by a black line and are presented with the standard error (SE). The graph illustrating the equilibrium responses (Req) as a function of enolase concentration (up to 400 nM) is presented in Supplementary materials (Figure 3).

80x49mm (300 x 300 DPI)

Figure 7. Mapping of the Als3 regions involved in enolase binding. (A) schematic representation of Als3 expressed on the surface of *S. cerevisiae* and corresponding deletion mutants, based on (Bamford et al., 2015), AFR – amyloid-forming region, GPI – glycosylphosphatidylinositol, HA – hemagglutinin; (B) *S. cerevisiae* cells (3×10^7 cells) expressing *C. albicans* adhesin Als3, specific deletion mutants of Als3, or the pBC542 vector alone, were incubated with *C. albicans* biotin-labeled enolase. The amount of bound protein was determined with the SA-HRP/TMB detection system. The values obtained with the vector alone control were subtracted from all other values. The experiment was performed in triplicate and a representative result is shown as the mean $\pm$ standard deviation (SD). Statistical significance levels were determined using one-way ANOVA with a Tukey’s multiple comparisons test relative to pBC-Als3; ** $p < 0.01$, *** $p < 0.001$.

Figure 8. Characteristics of the mutual interactions between the HK and HPG host proteins and Als3 and Eno1 on the yeast cell surface. (A-B, E-F) *S. cerevisiae* cells (5×10^6 cells per point) expressing surface Als3 lacking its amyloid fragment were incubated in suspension (50 μ L) with HPG or HK host proteins conjugated with fluorescein and over an increasing concentration range of enolase (Eno1) conjugated with Alexa Fluor 647. After 90 minutes of incubation and gentle cell washing, flow cytometry analysis was performed, with fluorometric detection conducted at excitation wavelengths of 488 nm or 633 nm and emission wavelengths 519 nm or 660 nm for fluorescein or Alexa Fluor 647, respectively. (C) The binding of the HK and HPG in the presence of Eno1 was analyzed in the same *S. cerevisiae* model using biotinylated host proteins and the SA-HRP/TMB detection system. (D) Identification of the Als3 regions important for these mutual interactions was performed using the *S. cerevisiae* surrogate for Als3 as described above and the SA-HRP/TMB detection system.

168x175mm (300 x 300 DPI)

Als3-mediated surface location of *C. albicans* Eno1 affects the interaction of fungal cells with host proteins

60x50mm (300 x 300 DPI)

**The Als3-mediated attachment of enolase on the surface of *Candida albicans* cells
regulates their interactions with host proteins**

Justyna Karkowska-Kuleta, Ewelina Wronowska, Dorota Satala, Marcin Zawrotniak, Grazyna
Bras, Andrzej Kozik, Angela H. Nobbs and Maria Rapala-Kozik*

Candida albicans enolase (Eno1) displayed mostly on the surface of fungal filamentous forms
attaches to the cell wall via adhesin from agglutinin-like sequence protein family - Als3.
Surface-displayed enolase plays an important role in fungal pathogenicity by mediating *C.*
albicans interactions with host proteins that are components of plasma proteolytic cascades
- plasminogen (HPG) and kininogen (HK).