Hyphal induction in the human fungal pathogen *Candida albicans* reveals a characteristic wall protein profile

Clemens J. Heilmann, Alice G. Sorgo, Adriaan R. Siliakus, Henk L. Dekker, Stanley Brul, Chris G. de Koster, Leo J. de Koning and Frans M. Klis

Correspondence

Frans M. Klis
F.M.Klis@uva.nl

Swammerdam Institute for Life Sciences, Universiteit van Amsterdam, Science Park 904, 1098XH Amsterdam, The Netherlands

The ability of *Candida albicans* to switch from yeast to hyphal growth is essential for its virulence. The walls and especially the covalently attached wall proteins are involved in the primary host–pathogen interactions. Three hyphal induction methods were compared, based on fetal calf serum, the amino sugar *N*-acetylglucosamine (GlcNAc) and the mammalian cell culture medium Iscove's modified Dulbecco's medium (IMDM). GlcNAc and IMDM were preferred, allowing stable hyphal growth over a prolonged period without significant reversion to yeast growth and with high biomass yields. We employed Fourier transform-MS combined with a ^{15}N -metabolically labelled reference culture as internal standard for relative quantification of changes in the wall proteome upon hyphal induction. A total of 21 wall proteins were quantified. Our induction methods triggered a similar response characterized by (i) a category of wall proteins showing strongly increased incorporation levels (Als3, Hwp2, Hyr1, Plb5 and Sod5), (ii) another category with strongly decreased levels (Rhd3, Sod4 and Ywp1) and (iii) a third one enriched for carbohydrate-active enzymes (including Cht2, Crh11, Mp65, Pga4, Phr1, Phr2 and Utr2) and showing only a limited response. This is, to our knowledge, the first systematic, quantitative analysis of the changes in the wall proteome of *C. albicans* upon hyphal induction. Finally, we propose new wall-protein-derived candidates for vaccine development.

Received 1 March 2011

Revised 13 April 2011

Accepted 16 May 2011

INTRODUCTION

The opportunistic fungal pathogen *Candida albicans* is a leading cause of fungal death in a clinical setting (Wisplinghoff *et al.*, 2004). In the immunocompetent population, *C. albicans* colonizes skin and mucosal surfaces which can lead to acute infections. Over 80% of all women experience at least one bout of vaginal candidiasis in their lifetime. In immunosuppressed patients, life-threatening bloodstream infections occur regularly and are often fatal if not diagnosed in time. In addition, the emergence of antifungal-resistant strains in hospitals around the world highlights the need for better treatment options and new antifungals and vaccines.

The cell wall of *C. albicans* is the initial site of host–pathogen interactions and is an obvious target for the development of antifungals and vaccines. It is composed of chitin, a glucan layer and mannoproteins. Most wall proteins are covalently linked to β -1,6-glucans by modified

glycosylphosphatidylinositol (GPI) anchors. The genome of *C. albicans* contains 115 predicted GPI-proteins that can be potentially linked to the wall (Richard & Plaine, 2007). Most of these proteins are only expressed under specific conditions and our previous work suggests that there are no more than approximately 20–30 wall proteins expressed at any given time (Klis *et al.*, 2010). GPI wall proteins generally show a modular structure with an N-terminal conserved domain followed by a highly *O*-glycosylated serine- and threonine- (S/T)-rich domain that can also include a repeat region (Klis *et al.*, 2009). In particular, mannosylation of wall proteins is important for limiting wall porosity (De Groot *et al.*, 2005) and might play a role in β -glucan masking (Wheeler *et al.*, 2008). Wall proteins show a variety of functions ranging from adhesion (Hoyer *et al.*, 1998; Staab *et al.*, 1999) and iron acquisition (Almeida *et al.*, 2008) to tissue invasion (Schaller *et al.*, 2005) and defence against the immune response (Frohner *et al.*, 2009). Recently, the response of the wall proteome to changes in ambient pH has been described (Sosinska *et al.*, 2011). The adhesins Als1 and Als3 as well as Hwp1 and Hyr1 have been used to vaccinate mice against *C. albicans* infections, resulting in reduced fungal burden and strongly improved survival (Luo *et al.*, 2010; Spellberg *et al.*, 2006).

Abbreviations: FCS, fetal calf serum; FT-MS, Fourier transform-MS; GlcNAc, *N*-acetylglucosamine; GPI, glycosylphosphatidylinositol; S/T-rich, serine- and threonine-rich.

Eight supplementary tables are available with the online version of this paper.

The yeast-to-hypha transition is the most prominent morphological change in the *C. albicans* life cycle. Hyphal cells are important for the formation of biofilms (Chandra *et al.*, 2001; Nobile & Mitchell, 2005; Nobile *et al.*, 2006) as well as the invasion of host tissues, while yeast cells are thought to be important for dispersal in the host. The transition also triggers the secretion of degrading enzymes (Naglik *et al.*, 2004) and the production of proteins that protect against oxidative stress (Martchenko *et al.*, 2004). Mutant strains locked in either the yeast or the hyphal morphotype have been shown to be avirulent (Lo *et al.*, 1997) and efficient transition from one stage to the other is important for full virulence.

The yeast-to-hypha transition is tightly regulated by a regulatory network, which includes among others the transcription factors Efg1, Cph1 and Tup1 (reviewed by Biswas *et al.*, 2007), which are activated by morphogenetic stimuli such as the presence of serum, the amino sugar *N*-acetylglucosamine (GlcNAc) (Simonetti *et al.*, 1974) or specific amino acids (Maidan *et al.*, 2005), as well as upon interaction with innate immune cells like macrophages or neutrophils (Lorenz *et al.*, 2004). Transcriptional studies have indicated that on the transcript level, the yeast-to-hypha transition leads to numerous changes in the expressions of genes encoding proteins involved in both intracellular processes and the composition of the cell surface (Kadosh & Johnson, 2005; Nantel *et al.*, 2002; Sohn *et al.*, 2003). However, transcriptional studies only describe an upregulation or downregulation of gene expression and not the abundance of the final protein. To date the comparison of genome-wide transcription and proteomic studies is limited (Monteoliva *et al.*, 2011) and our understanding of the proteome dynamics is still in its infancy.

In this study we present a comprehensive proteomic analysis based on highly accurate mass determination by Fourier transform (FT)-MS and the use of an internal standard consisting of ^{15}N -metabolically labelled wall proteins for the relative quantification of the changes in the wall proteome of *C. albicans* upon the yeast-to-hypha transition. We show that hyphal induction triggers a specific response of the wall proteome independent of the induction method. In addition, we identify four categories of wall proteins, depending on morphotype and growth temperature. Many of the quantified proteins promise to be useful for diagnostic and therapeutic purposes.

METHODS

Strains, growth conditions and induction of hyphal growth.

All chemicals were obtained from Sigma-Aldrich unless otherwise stated. *C. albicans* SC5314 (Gillum *et al.*, 1984) was pre-cultured in liquid yeast peptone dextrose (YPD) medium (10 g yeast extract l^{-1} , 20 g peptone l^{-1} and 20 g glucose l^{-1}) in a rotary shaker at 200 r.p.m. and 30 °C overnight. The next day the overnight culture was used to inoculate flasks containing 50 ml YNB-S [6.7 g yeast nitrogen base (YNB) l^{-1} , 20 g sucrose l^{-1} , 75 mM 3-(*N*-morpholino)-2-hydroxypropanesulphonic acid (MOPSO), pH 7.4] at OD_{600} 0.5, which corresponds to an initial dry weight of 0.15 mg ml^{-1} (the dry

weight was determined by drying the cell pellet of a 50 ml sample overnight at 65 °C in a pre-weighed centrifuge tube). The cultures were incubated for 18 h at either 30 or 37 °C and 200 r.p.m.

Hyphal growth was induced by supplementing YNB-S with either 5 mM GlcNAc or 10 % fetal calf serum (FCS), or by using 2 % proline as sole carbon source. In addition, *C. albicans* was incubated in the cell culture media Iscove's modified Dulbecco's medium (IMDM-S), α -modified minimum essential medium (α -MEM-S) or Roswell Park Memorial Institute medium 1640 (RPMI 1640-S) at 37 °C overnight [all supplemented with 75 mM MOPSO, pH 7.4, and 20 g sucrose l^{-1} (sucrose supplement indicated by "-S")].

Metabolic ^{15}N -labelling of the reference cultures. To ensure maximal ^{15}N -loading, *C. albicans* cells from a pre-culture were used to inoculate a second pre-culture to OD_{600} 0.05 in YNB-S with ^{15}N -labelled ammonium sulfate as the sole nitrogen source (Spectra Stable Isotopes; ^{15}N content >99 %) and buffered with 75 mM tartaric acid to pH 4. Tartaric acid was used because it has two pK_a values close to pH 4 (4.37 and 3.02 at 37 °C) and is not metabolized by *C. albicans* (our observations). The culture was incubated overnight at 30 °C and 200 r.p.m. This ^{15}N -labelled pre-culture was used to inoculate two 600 ml cultures of ^{15}N -labelled YNB-S, either buffered with 75 mM tartaric acid at pH 4 or with 75 mM MOPSO at pH 7.4, to OD_{600} 0.1. The cultures were incubated at 37 °C and 200 r.p.m. for 18 h. The ^{15}N -labelled cells of both cultures were harvested and combined 1:1; their walls were isolated, divided into aliquots, freeze-dried and stored at -80 °C.

Cell wall preparation. The cell pellet was collected by centrifugation, frozen in liquid nitrogen and ground into a fine powder with a pestle and mortar. This step ensured highly efficient breakage, especially in the case of hyphal growth, and reduced the amount of cytosolic contaminants in the final sample. Cell walls were prepared as described elsewhere (de Groot *et al.*, 2004). Briefly, the finely ground cell pellet was washed several times with PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 per l; pH 7.4 with HCl) and then subjected to breakage in a FastPrep bead beater (Savant Instruments) with glass beads (0.25–0.50 mm, 12–16 runs for 45 s at speed 6.0) in the presence of a protease inhibitor cocktail. Full breakage was controlled by light-microscopic inspection. The pellet was washed several times with 1 M NaCl and stored overnight at 4 °C. The following day, the pellet was washed several times with MilliQ water and then boiled four times for 10 min in SDS extraction buffer [150 mM NaCl, 2 % (w/v) SDS, 100 mM Na-EDTA, 100 mM β -mercaptoethanol, 50 mM Tris/HCl, pH 7.8], washed with MilliQ water and lyophilized overnight. The resulting purified wall pellets were either stored at -80 °C until needed or directly reduced and S-alkylated (Yin *et al.*, 2005). The wall pellets were treated with reducing solution (10 mM dithiothreitol in 100 mM NH_4HCO_3) and incubated for 1 h at 55 °C. After centrifugation the supernatant was replaced by alkylating solution (65 mM iodoacetamide in 100 mM NH_4HCO_3) for 45 min at room temperature in the dark. Alkylation was stopped by incubating the samples in 55 mM dithiothreitol/100 mM NH_4HCO_3 for 5 min. Subsequently, samples were washed six times with 50 mM NH_4HCO_3 and either frozen in liquid nitrogen and stored at -80 °C or directly used for experiments.

$^{14}\text{N}/^{15}\text{N}$ mixing and sample preparation for MS analysis.

Our quantification workflow is summarized in Fig. 1(a). The freeze-dried, reduced and alkylated walls of both the ^{14}N -query and the ^{15}N -reference walls were weighed and resuspended in 50 mM ammonium bicarbonate buffer and mixed to obtain a 1:1 mixture based on dry weight (2 mg:2 mg). The mixed walls were digested for 18 h using 2 μg Trypsin Gold (Promega) and subsequently desalted using a C18 tip column (Varian). The amount of peptides was determined using a NanoDrop ND-1000 (Isogen Life Science) at

Fig. 1. (a) Workflow to obtain both peptide passports and ^{14}N : ^{15}N -peptide pair ratios of the ^{14}N -query culture with respect to the ^{15}N -labelled reference culture. The ^{15}N internal standard is cancelled out when two ^{14}N -query cultures are compared with each other. (b) Representative comparison of a hyphal induction condition with a non-induced control. Positive values for a protein indicate a strong increase in abundance upon hyphal induction and vice versa.

205 nm as described before (Desjardins *et al.*, 2009; Sorgo *et al.*, 2010). For each run a sample containing 0.8 μg peptides was used.

MS analysis and data processing. Accurate mass data were acquired using an ApexQ FT ion cyclotron resonance mass spectrometer (Bruker Daltonik) equipped with a 7T magnet and a CombiSource coupled to an Ultimate 3000 (Dionex) HPLC system with a PepMap100 C18 (5 μm particle size, 10 nm pore size, 300 μm inner diameter $\times$ 5 mm length) precolumn and a PepMap100 C18 (5 μm particle size, 10 nm pore size, 300 μm inner diameter $\times$ 250 mm length) analytical column (Dionex).

Samples (3 μl) containing 0.8 μg of tryptic peptides in a 0.1% trifluoroacetic acid aqueous solution were loaded onto the pre-column. Following injection, a linear gradient (from 0.1% formic acid/100% H_2O to 0.1% formic acid/40% $\text{CH}_3\text{CN}/60\%$ H_2O) was applied over a period of 120 min at a flow rate of 3 $\mu\text{l min}^{-1}$. During elution a chromatogram of up to 1850 high-resolution electrospray ionization-FT-MS spectra was recorded using an MS duty cycle of about 3 s.

The data were processed using the Data Analysis 3.4 software program (Bruker Daltonik). The 1800 mass spectra were batch-wise extracted from the chromatogram, and the monoisotopic masses of the peptides were determined using Bruker's peak recognition technology SNAP II. Mass calibration was achieved by selective extraction and subsequent summation of 12 mass spectra from the chromatogram corresponding to MSMS/MASCOT identified tryptic peptides originating from wall proteins (see Supplementary Tables S1–S2, available with the online version of this paper). With the calculated masses of these calibrant peptides the summed spectrum was mass calibrated and the resulting calibration parameters were applied to all spectra in the chromatogram. This resulted in a mass calibration of better than 1.5 p.p.m. over the entire chromatogram for all analyses.

For each FT-MS analysis the resulting array of up to 1800 monoisotopic mass lists was exported as a MASCOT generic file (.mgf). Ion abundances in the exported array of monoisotopic mass lists were the spectral intensities of the most abundant isotope summed over all charge states for each peptide. The exported .mgf file was imported into the in-house-developed CoolToolBox software program (de Koning *et al.*, 2006; Heijnis *et al.*, 2011; Kasper *et al.*, 2007; Müller *et al.*, 2009; Popolo *et al.*, 2008; Sosinska *et al.*, 2011). From the imported array of up to 1800 monoisotopic mass spectra the CoolToolBox program constructed up to 1000 peptide ion chromatograms. For each peptide ion chromatogram the mass and retention time were determined at the apex of the chromatogram profile and the abundance was summed over the ion chromatogram profile.

The Ultimate liquid chromatography (LC)-MS data processing resulted in a peptide monoisotopic mass list with corresponding abundances and LC retention times. Peptide assignments of the ion masses were obtained by matching the processed LC-FT-MS data from the tryptic peptides within a mass window of 1.5 p.p.m. with the masses of tryptic peptides from corresponding ^{14}N - and ^{15}N - *C. albicans* wall proteins, obtained from an *in silico* digestion of the *C. albicans* database of 153 potential processed wall proteins (Sorgo *et al.*, 2010). The peptide elution profiles were shorter than 25 s. As expected the retention behaviour of the ^{14}N - and ^{15}N -peptides was similar, with the ^{15}N -peptides consistently eluting only a few seconds before the corresponding ^{14}N -peptides. Using this criterion combined with the accurate masses of both ^{14}N - and ^{15}N -peptides, the CoolToolBox program automatically picked out all $^{14}\text{N}/^{15}\text{N}$ -peptide pairs using a peptide pair retention time window of only 10 s over the total gradient of 120 min. This resulted in unique series of assigned tryptic peptide pairs with the corresponding $^{14}\text{N}/^{15}\text{N}$ isotopic ratios for all identified wall proteins (see Supplementary Tables S3–S7,

available with the online version of this paper). Further validation of the $^{14}\text{N}/^{15}\text{N}$ peptide pair assignments was obtained by matching their retention times with the retention times of the corresponding peptides identified with a LC-FT-MS-MS/MASCOT analysis of tryptic digests of a *C. albicans* ^{14}N -proteome obtained under identical experimental LC conditions.

For each identified wall peptide pair the CoolToolBox program searched the *in silico* digest tryptic peptide database generated from the ^{14}N - and ^{15}N -wall proteome database for possible alternative assignments within a mass window of 1.5 p.p.m. For (i) identical peptide sequences in other proteins, for (ii) alternative peptide sequences with the same number of nitrogen atoms and for (iii) alternative peptide sequences with a different number of nitrogen

atoms. The numbers of each of the three alternative assignments are listed in the comprehensive Supplementary Tables S3–S7. These numbers indicate the uniqueness of the peptide pairs used for protein quantification. At least three biological replicates of all conditions were measured (Supplementary Tables S3–S7). After analysing the runs as described above, 21 proteins were quantified for all samples (Tables 1 and 3). To evaluate the accuracy of our measurements, the biological relative standard error (RSE) was calculated by averaging the RSE for each protein over all biological replicates. The biological variation was in the range of 18–45 % (Table 3) with a median value of 27 %. We quantified between one and 13 peptide pairs per protein (Table 3). For a complete list of all identified peptide pairs see Supplementary Table S8 (available with the online version of this paper).

Table 1. Wall-associated secretory proteins and their characteristics

Undef., Undefined.

Name	Function	Protein information*					Peptide location†	Coverage CD (%)‡
		AA	SP	CD	S/T	ω		
GPI proteins								
Als1§	Adhesin	1260	1–17	18–299	~330–GPI	1239	19–254	~11–19
Als2§	Adhesin	2362	1–17	18–299	~330–GPI	II	19–310	~23
Als3	Adhesin, ferritin receptor	1155	1–17	18–299	~330–GPI	1134	19–316	~31–54
Als4	Adhesin	2100	1–17	18–299	~330–GPI	2079	19–291	~13–28
Cht2	Chitinase, GH18#	583	1–19	20–303	~300–GPI	560	99–299	~40–43
Crh11	Transglycosylase, GH16	453	1–21	22–275	~280–GPI	430	29–246	~36–46
Ecm33	Role in wall integrity	423	1–18	19–357	~350–GPI	400	139–341	~20–28
Hwp2	Unknown	908	1–20	Undef.	Undef.	887	21–25	
Hyr1	Exo- α -sialidase-like	919	1–20	211–335	~350–GPI	895	250–260	~8
Pga4	Transglycosidase, GH72	451	1–18	19–322	~370–GPI	430	69–385	~26–41
Phr1	Transglycosylase, GH72	548	1–20	21–477	~480–GPI	516	21–452	~29–41
Phr2	Transglycosylase, GH72	544	1–22	23–464	~480–GPI	515	23–438	~4–13
Plb5	Phospholipase	754	1–19	96–754	Undef.	726	120–123	~1
Rbt1	Unknown, Flo11 CD	721	1–20	86–198	~270–GPI	700	75–234	~38–68
Rbt5§	Iron acquisition	241	1–20	47–112	~120–GPI	221	47–92	~49
Rhd3	Unknown, Flo11 CD	204	1–15	Undef.	None	182	16–150	
Sap9	Yapsin-like protease	544	1–17	19–519	~480–GPI	520	294–439	~2–11
Sod4	Superoxide dismutase	232	1–15	27–172	~170–GPI	209	40–117	~9–14
Sod5	Superoxide dismutase	228	1–15	27–171	~170–GPI	205	26–140	~12–42
Ssr1	Wall structure, CFEM	234	1–22	23–84	~80–GPI	215	23–79	~68
Utr2	Transglycosylase, GH16	470	1–23	30–309	~370–GPI	440	75–310	~25–33
Ywp1	Role in host dispersal	533	1–21	Undef.	~160–410	511	87–339	
Non-GPI proteins								
Fwp1§	Fluconazole induced	324	1–25	Undef.	Undef.	None	236–306	
Mp65	Transglycosylase, GH17	378	1–17	127–378	~60–110	None	142–363	~24–35
Pir1	Glucan cross-linking	346	1–18	174–346	None	None	249–346	~28–33

*AA, Length of the protein in amino acids; SP, predicted signal peptide [SignalP3.0 (Emanuelsson *et al.*, 2007)]; CD, conserved domain [CGD-CDART (Skrzypek *et al.*, 2010)]; S/T, serine/threonine-rich domain; ω , predicted GPI-anchor attachment site [Big-PI Fungal predictor (Eisenhaber *et al.*, 2004)].

†Region of tryptic peptides. The identified peptides are listed in Supplementary Tables S3–S8.

‡CD (%), sequence coverage (%) of the conserved domain.

§Peptide pairs were not consistently detected for Als1, Als2, Rbt5 and Fwp1 (Orf19.7104) (Supplementary Tables S3–S7) and were therefore not used for quantitative, comparative analysis in Table 3.

||Als2 is described as a GPI-anchored protein but has no predicted GPI-anchor attachment site according to the Big-PI Fungal predictor (Eisenhaber *et al.*, 2004).

#Glycoside hydrolase family no. [CAZy database (Cantarel *et al.*, 2009)].

RESULTS

Relative quantification method for wall proteins

A metabolically labelled ^{15}N -reference culture enables relative quantification of unlabelled ^{14}N -query cultures. These can originate from highly different growth conditions and sources such as planktonic cultures, biofilms, and infected tissues and organs, irrespective of nitrogen sources. To obtain a wide representation of wall proteins, and since the culturing pH is known to have a strong effect on the mode of growth and the composition of the wall proteome (Sosinska *et al.*, 2011), we used an acidic pH (pH 4) and a pH close to neutral (pH 7.4) for metabolic ^{15}N -labelling of *C. albicans*. This allows for systematically collecting query:reference ratios of individual wall proteins belonging to wall proteomes obtained in this and later studies and for a future meta-analysis of the data. To ensure maximal ^{15}N -loading of the cells, we grew them in two consecutive overnight cultures with the stable nitrogen (^{15}N) isotope present in the form of ammonium sulfate (Oda *et al.*, 1999) as sole nitrogen source. We used tryptic shaving of the cell wall (Yin *et al.*, 2005) for optimal release of covalently anchored wall proteins. The high FT-MS mass accuracy of better than 3 p.p.m. together with a narrow elution time window allowed us to set strict rules for the identification of proteins. Our mass spectrometric analysis was dominated by a very abundant peptide (IYDQLPECAK) that was derived from three proteins (Csa1, Pga10 and Rbt5), making it unsuitable for quantification (see discussion below).

Wall protein characteristics and coverage

Table 1 shows that we identified 25 wall-associated proteins with an N-terminal signal peptide in total, 22 of which are predicted to be GPI-anchored. Under our culture conditions we did not consistently detect peptide pairs for the GPI proteins Als1, Als2 and Rbt5 and the non-GPI-protein Fwp1 (Orf19.7104) (Supplementary Tables S3–S7). For example, we found that the level of Als1 was increased approximately fivefold in the walls of cultures grown in the presence of the hyphal inducers fetal calf serum or GlcNAc compared with the reference walls, consistent with its presence in the hyphal base (Coleman *et al.*, 2010) (Supplementary Tables S5 and S6). However, it could not be identified with certainty in the other cultures. The remaining subset of 21 secretory proteins, 19 of which were GPI proteins, was used for further quantitative, comparative analysis. Most of the GPI-proteins have a conserved domain in the N-terminal region, which is also the origin of almost all of the tryptic peptides we detected. Standard mass spectrometric techniques cannot detect all known covalently linked wall proteins. The tryptic peptides can be either too small or too large for the m/z window used as is the case for the tryptic peptides in the N-terminal domain of Hwp1 (Candida Genome Database) (Skrzypek *et al.*, 2010; Sosinska *et al.*, 2011). Glycosylation can interfere

with their identification as frequently happens in case of the C-terminal part of GPI-proteins, which is S/T-rich and predicted to be highly glycosylated. Chemical deglycosylation can partially solve this problem as shown for Pga59 and Pga62 (Castillo *et al.*, 2008), but the currently available deglycosylation procedures have ill-defined side-effects. This results in less efficient and reproducible identification of wall proteins and prevents their quantification (our unpublished data). Furthermore, as mentioned in the previous section, identification is sometimes partial, when only a single tryptic peptide is identified that belongs to several members of the same wall protein family.

Under all growth conditions we identified two predicted cytosolic proteins in our wall preparations even after extensive extraction with hot SDS, namely Ssa2 and Tdh3 (Supplementary Tables S3–S7). Ssa2 is a chaperone protein belonging to the Hsp70 family, and evidence has been presented previously that it is present at the hyphal surface (Urban *et al.*, 2003). We found it also in the cell walls of cultures grown at 30 °C and thus completely in the yeast form. Tdh3 is a glycolytic enzyme and has been detected at the cell surface of both yeast cells and hyphae (Gil-Navarro *et al.*, 1997). Since they lack a classical signal peptide, it is not known how these two proteins are retained by the walls and how they reach this location.

Selection of three representative methods for hyphal induction

A variety of conditions induce the yeast-to-hypha transition in *C. albicans*. We previously showed that culturing cells in the presence of sucrose instead of glucose simulates a low-glucose extracellular environment while allowing high biomass yields (Sorgo *et al.*, 2010). While *C. albicans* remained in the yeast form at 30 °C even at a hyphal-permissive pH of 7.4, a shift to 37 °C induced some hyphal

Table 2. Biomass and degree of hyphal formation after 18 h

Condition	Growth temperature (°C)	Biomass (mg ml ⁻¹ ; SEM, n=3)	Hyphal formation*
YNB-S	30	7.6 ± 0.11	–
YNB-S	37	5.0 ± 0.24	+
YNB-S + 10 % FCS	37	6.9 ± 0.24	+
YNB + 2 % Proline	37	2.5 ± 0.08	+
RPMI 1640-S	37	4.1 ± 0.26	++
α-MEM-S	37	4.9 ± 0.42	++
YNB-S + 5 mM GlcNAc	37	6.4 ± 0.08	+++
IMDM-S	37	5.1 ± 0.09	+++

*–, No hyphae; +, moderate hyphal formation; ++, intermediate hyphal formation; + + +, strong hyphal formation.

Table 3. Wall protein ratios of *C. albicans* grown in various media in relation to an internal standard culture of ¹⁵N-wall proteins

¹⁵N denotes that peptides that were suitable for quantification were only present in the reference culture, whereas ¹⁴N denotes that such peptides were only present in the query culture.

Protein	YNB-S 30 °C	YNB-S 37 °C	YNB-S 10 % FCS 37 °C	YNB-S 5 mM GlcNAc 37 °C	IMDM-S 37 °C	RSE%* (average)	No. of peptide pairs quantified
Yeast-associated proteins							
Rhd3	1.12	0.54	0.52	0.19	0.03	27	2–11
Sod4	7.06	9.50	0.58	1.96	0.11	30	1–2
Ywp1	1.41	1.95	0.82	0.05	0.03	25	1–3
Low-temperature-associated proteins							
Als4	77.0	0.96	0.37	2.08	3.30	45	2–5
Pir1†	0.28	0.05	0.06	0.02	0.04	28	2–3
Morphotype-independent proteins							
Cht2	2.91	1.03	1.26	0.77	2.35	21	7–8
Crh11	0.97	1.10	1.65	1.68	1.01	22	8–11
Ecm33	0.57	0.47	0.66	0.23	0.19	21	6–7
Mp65†	0.48	0.69	1.33	0.31	0.27	26	4–7
Pga4	0.51	0.63	0.85	0.51	0.47	28	6–9
Phr1	1.14	2.47	2.39	3.14	5.13	23	9–13
Phr2	0.39	0.23	0.18	0.20	0.15	26	1–4
Rbt1	¹⁵ N	0.62	0.25	2.25	0.42	18	3–5
Sap9	2.10	1.49	1.11	0.84	5.42	32	1–5
Ssr1	0.93	1.17	1.52	0.73	0.44	28	3
Utr2	0.74	1.33	1.68	0.61	0.32	29	6–8
Hypha-associated proteins							
Als3	0.06	0.83	1.19	3.93	9.10	23	4–12
Hwp2	¹⁵ N	0.71	1.78	4.89	17.2	26	1
Hyr1	¹⁵ N	9.05	58.6	560	¹⁴ N		1
Plb5	¹⁵ N	0.77	1.16	7.40	9.33	31	1
Sod5	¹⁵ N	0.97	5.42	6.00	23.9	23	2–5

*RSE%, relative standard error.

†Non-GPI protein.

formation. All growth conditions were tested for biomass formation after 18 h of incubation with an initial dry weight of 0.15 mg ml⁻¹ (Table 2). At 30 °C 7.6 mg biomass ml⁻¹ was obtained after 18 h, whereas at 37 °C the biomass yield was reduced to 5.0 mg ml⁻¹. FCS has been the induction method of choice for decades but due to its animal origin it is not completely defined and varies from batch to batch. Over the 18 h incubation period used in this study in YNB-S+10 % FCS (6.9 mg ml⁻¹), reversion to the yeast form was common. Adding 5 mM GlcNAc (6.4 mg ml⁻¹) to a culture led to strong hyphal induction with more than 90 % hyphae after 18 h. We also tested YNB with 2 % proline as sole carbon source but discarded this induction method because of its low biomass yield (2.5 mg ml⁻¹). To mimic physiological conditions more closely, we tested three FCS-free media regularly used for mammalian cell culture: IMDM-S (5.1 mg ml⁻¹), α -MEM-S (4.9 mg ml⁻¹) and RPMI 1640-S (4.1 mg ml⁻¹). These media maintain hyphal growth even when entering stationary phase with IMDM-S showing the strongest hyphal induction. Based on these results we selected the following hyphal induction media for further study:

YNB-S+10 % FCS and two fully synthetic media, namely, YNB-S+5 mM GlcNAc and IMDM-S.

Of the three induction methods used for quantification, FCS showed the weakest hyphal induction after 18 h judging by both visual inspection (Table 2) and the abundance of hyphal indicator proteins (Table 3). FCS is usually not used for long-term induction, because of increasing reversion to the yeast form in time. GlcNAc at millimolar concentrations was shown to be a strong inducer of hyphal growth even for a prolonged period of time. IMDM-S was the strongest hyphal inducer in our study. The abundance of proteins related to hyphal growth (Als3, Hwp2, Plb5, Sod5 and especially Hyr1) was strongly increased in IMDM-S-induced walls while levels of yeast-associated protein (Sod4, Rhd3 and Ywp1) were strongly decreased (Table 3). IMDM-S-grown cells showed, in addition to these proteins, an increase of Cht2, Phr1 and Sap9. A third category of proteins showed relatively limited variation in abundance under all induction conditions. This category mainly consists of proteins involved in cell wall maintenance and remodelling (e.g. Crh11, Ecm33, Mp65, Pga4, Phr2, Ssr1 and Utr2). Conceivably, these

proteins serve a core function required for both yeast and hyphal growth and therefore are required to be maintained at a certain level. Fig. 1(b) shows a relative comparison of protein abundances in IMDM-S at 37 °C with YNB-S at 37 °C. The separation into hypha-associated, yeast-associated and morphotype-independent proteins is representative of all inducers (see also Table 3).

The wall proteome response of IMDM- and GlcNAc-grown cells is highly similar

Ranked correlation analysis (Lehmann & D'Abrera, 1975) of their quantified wall proteomes revealed a strong positive correlation [Spearman's rank correlation coefficient (R_s)=0.88; $P=1 \times 10^{-7}$]. These data suggest that the wall proteome response is mainly dependent on the presence, but not the character, of a hyphal inducer. This is supported by the observation that also the combination of FCS/IMDM and FCS/GlcNAc showed a significant positive correlation (R_s =0.67, P =0.001 and R_s =0.57, P =0.008, respectively). Consistent with the considerable reversion to the yeast form and the low relative abundance of hyphal indicator proteins, FCS is the only inducer showing a significant correlation with YNB-S at 37 °C (R_s =0.56, P =0.01), while both GlcNAc and IMDM-S are not significantly correlated with YNB-S at 37 °C.

Identification of three yeast-indicating, five hypha-indicating and two low-temperature-associated proteins

Our data show that Als3, Hwp2, Hyr1, Plb5 and Sod5 are good indicators of hyphal growth (Table 3) as also suggested by earlier transcriptional studies (Argimón *et al.*, 2007; Hayek *et al.*, 2010; Kadosh & Johnson, 2005; Theiss *et al.*, 2006). These proteins increased upon the temperature change from 30 to 37 °C and increased even more when an inducer was added, showing morphotype association. Rhd3, Sod4 and Ywp1 are indicative of yeast cells because they showed the inverse relationship as indicated by earlier studies concerning Rhd3 and Ywp1 (de Boer *et al.*, 2010; Granger *et al.*, 2005). On the other hand, Als4 and Pir1 levels were sharply decreased upon increasing the temperature from 30 to 37 °C and did not change significantly when inducers were added, indicating association with low temperature (Table 3). These results agree qualitatively with transcriptional data, which show that the expression of *ALS4* (Zhao *et al.*, 2005) and *PIR1* (Sohn *et al.*, 2003) decreases strongly upon switching the temperature from 30 to 37 °C. Pir1 is essential in *C. albicans* and is involved in wall regeneration and wall organization (Martínez *et al.*, 2004) and probably also in β -1,3-glucan cross-linking (Ecker *et al.*, 2006), while Als4 is important for the infection of buccal epithelial cells (Green *et al.*, 2004). Interestingly, these proteins have also been shown to be contact-dependent, suggesting that they are involved in infections of the skin, where the body temperature is generally lower (Sorgo *et al.*, 2011).

DISCUSSION

Although transcript levels are expected to correlate with protein synthesis rates, it seems rather unlikely that they show a linear correlation with actual protein levels, especially, when the environmental conditions are unstable. Transcript levels are thus expected to provide information about the direction in which the incorporation levels of wall proteins will move but to be much less valuable in predicting the actual changes in protein levels. A variety of studies have shown morphotype-specific changes in mRNA levels in response to hyphal inducers (Braun *et al.*, 2000; Braun & Johnson, 2000; Kadosh & Johnson, 2005; Sohn *et al.*, 2003). However, there are only a few quantitative proteomic studies available to complement these data (Monteoliva *et al.*, 2011). We set out to perform relative quantification of the covalently anchored cell wall proteins of *C. albicans* with and without induction of hyphal growth. We used a gel-free approach combining a ^{15}N -metabolically labelled standard culture and FT-MS with a mass accuracy of better than 3 p.p.m. over a large dynamic range. In this way, we were able to perform relative quantification of 21 *C. albicans* wall proteins.

We used three representative methods of hyphal induction. Muramyl dipeptides, the main inducing component of FCS (Xu *et al.*, 2008) and GlcNAc are both derivatives of peptidoglycan and strong inducers of hyphal growth (Alvarez & Konopka, 2007; Simonetti *et al.*, 1974). Hyphal induction by GlcNAc, acting as a signalling molecule, is mainly mediated by the activation of adenylyl cyclase (Castilla *et al.*, 1998), similar to muramyl dipeptides (Xu *et al.*, 2008). IMDM-S, α -MEM-S and RPMI 1640-S contain all amino acids and some vitamins and are all good inducers, with methionine and proline being the most probable cause of induction (Maidan *et al.*, 2005). IMDM-S was preferred because it was a strong inducer and because the cultures remained in the hyphal growth mode until stationary phase, producing large amounts of biomass. Intriguingly, the three selected inducers, although very different, led to a highly similar response of the wall proteome to GlcNAc and IMDM and to a lesser extent also to FCS, which was due to its partial reversion to yeast growth.

Hypha-associated proteins are important for establishing and maintaining an infection

The adhesin Als3 (Argimón *et al.*, 2007), Hwp2 (Sohn *et al.*, 2003), Hyr1 (Bailey *et al.*, 1996), a potential exo- α -sialidase, the fungal-specific phospholipase Plb5 (Theiss *et al.*, 2006) and the superoxide dismutase Sod5 (Martchenko *et al.*, 2004) were identified as indicators of hyphal growth. Strikingly, all these proteins have infection-related roles ranging from adhesion and aggregation [Als3 (Hoyer *et al.*, 1998), Hwp2 (Alsteens *et al.*, 2010; Hayek *et al.*, 2010; Otoo *et al.*, 2008; Ramsook *et al.*, 2010)], homo- and heterologous biofilm formation [Als3 (Chandra *et al.*, 2001; Nobile & Mitchell, 2005; Nobile *et al.*, 2006; Silverman *et al.*, 2010), Hyr1 (Nobile *et al.*, 2006)],

facilitated endocytosis via cadherins [Als3 (Phan *et al.*, 2007)], iron acquisition from the host protein ferritin [Als3 (Almeida *et al.*, 2008)], tissue destruction and *in vivo* organ colonization [Plb5 (Ibrahim *et al.*, 1995; Theiss *et al.*, 2006)] as well as resistance to host defences. Hyr1 confers resistance to neutrophil killing (Luo *et al.*, 2010), while Sod5 is important for coping with the extracellular reactive oxygen species produced by bone-marrow-derived macrophages, myeloid dendritic cells (Frohner *et al.*, 2009), neutrophils and other granulocytes (Fradin *et al.*, 2005). All these features are important for mounting an effective infection and their association with hyphal growth hints at their importance during localized, invasive infections.

Yeast-associated proteins play a role in dissemination and systemic infections

Rhd3, Sod4 and Ywp1 were indicative of yeast growth. For Rhd3 and Ywp1 no functions have yet been described while Sod4 is a superoxide dismutase. Rhd3 is involved in establishing a systemic infection (de Boer *et al.*, 2010). *RHD3* knockouts showed a significant decrease in cell wall mannans and resulted in reduced cytokine production in reconstituted human epithelium infections (de Boer *et al.*, 2010), which implies a role in immune evasion. This might be directly related to the morphotype-specific unmasking of β -glucans (Wheeler & Fink, 2006; Wheeler *et al.*, 2008). Ywp1 abundance is strongly decreased in hyphal walls (our data) as is also indicated by transcriptional studies (Sohn *et al.*, 2003) (Table 3). It seems to play a role in dispersal in the host, since knockouts adhere and form biofilms more readily (Granger *et al.*, 2005). It also possesses a propeptide that might be useful for diagnostic purposes (Granger *et al.*, 2005). The abundance of Sod4, a functional homologue of Sod5, is inversely correlated with the degree of hyphal formation (Fradin *et al.*, 2005; Frohner *et al.*, 2009).

Morphotype-independent proteins serve housekeeping functions of the wall

Under all induction conditions analysed Cht2, Crh11, Ecm33, Mp65, Pga4, Phr1, Phr2, Ssr1 and Utr2 remained relatively stable in abundance. Intriguingly, they are all involved in cell wall maintenance and remodelling (de Groot *et al.*, 2004). This result is also interesting because it mirrors our observations of the secretome (Sorgo *et al.*, 2010). It should be noted that this set of proteins is stable in abundance with respect to the yeast-to-hypha transition but can be significantly affected by other stresses like azole treatment (Sorgo *et al.*, 2011). Remarkably, the transglycosylase Mp65 is present in both the core set defined for the secretome (Sorgo *et al.*, 2010) and every wall preparation and is largely morphotype-independent. This is in agreement with the observation that transcript levels of Mp65 are stable as well (Sohn *et al.*, 2003). Interestingly, it is not covalently anchored to the wall by any known linkages, raising the question of whether it becomes

trapped in the walls during homogenization. Furthermore, Mp65 is already established as a diagnostic biomarker and being immunodominant (Bromuro *et al.*, 1994; Gomez *et al.*, 1996). Its strong immunogenicity and high abundance make it a promising target for vaccine development. Finally, *PHR1* and *PHR2* are a classical example of pH-controlled wall-protein-encoding genes. They belong to the same family and share a similar function, with *PHR1* being strongly expressed at neutral pH and *PHR2* strongly expressed at pH <5.5 (De Bernardis *et al.*, 1998). The presence of Phr2 in the walls of cultures grown at neutral pH has been described previously (Sosinska *et al.*, 2011). However, at neutral pH its incorporation level was 36-fold lower than at pH 4, suggesting low wall levels at neutral pH. The consistently lower coverage (4–13 %) of the conserved domain of Phr2 compared with Phr1 (29–41 %) in our experiments, which have all been carried out at pH 7.4 (Table 1), supports this.

New targets for antifungal strategies, vaccine development and early diagnostics

The diverse functions of Als3 make it a promising target for the development of new antifungals and vaccines. Monoclonal antibodies bind to the N-terminal region of Als3 (Brena *et al.*, 2007). Vaccination of immunocompetent mice with the recombinant N terminus of Als3 significantly improved survival (Spellberg *et al.*, 2006). Similarly, vaccination with the N terminus of Hyr1 is immunoprotective in mice (Luo *et al.*, 2010). Our data suggest that the N terminus of Hwp2, Plb5 and Sod5, which are all GPI proteins and are therefore predicted to have their N-terminal region extended into the medium (Klis *et al.*, 2009), might be used in a similar fashion. All the peptides we identified also originate from the N-terminal region, which extends away from the cell wall, and might therefore be used in a peptide-based vaccine. The highly abundant peptide IYDQLPECAK is the beginning of the CFEM domain (Kulkarni *et al.*, 2003), a conserved domain of eight cysteine residues spaced over about 70 amino acids that has been implicated in biofilm formation (Pérez *et al.*, 2006). The cysteine in the peptide is the first of eight cysteines in the CFEM domain, which is present in five surface proteins in *C. albicans* (Csa1, Csa2, Pga7, Pga10 and Rbt5) and in a divergent form in Ssr1. Rbt5, Pga10 and Csa1 have been shown to be involved in iron acquisition (Weissman & Kornitzer, 2004; Weissman *et al.*, 2008). The CFEM domain is located in their N-terminal region and is not glycosylated, suggesting that it might be accessible on the surface. While all CFEM proteins except for Csa1 have only one copy of this domain, Csa1 has four, indicating a domain expansion. CFEM proteins are also present in other *Candida* species. The abundance of this particular peptide and the CFEM domain in general make them promising targets for marker and vaccine development.

In conclusion, our data give a first quantitative proteomic snapshot of the changes in the wall proteome during the yeast-to-hypha transition of *C. albicans*. Our method can be

easily expanded to other fungi, plants and micro-organisms. The use of a metabolically labelled reference culture as an internal standard allows (i) the comparison of diverse conditions without having to label them individually and (ii) the generation of a library of wall protein ratios under these conditions. We could further show that very different inducers result in a similar response of the wall proteome. In addition, we identified potential targets, especially in the N-termini of wall proteins, for the development of novel diagnostics as well as for a peptide-based vaccine.

ACKNOWLEDGEMENTS

We thank two anonymous reviewers for their valuable comments. We also thank Winfried Roseboom for technical assistance. F.M.K. is supported by the EU Programme FP7-214004-2 FINSysB. A. G. S. and C. J. H. are grateful for the support by all members of the FINSysB consortium.

REFERENCES

- Almeida, R. S., Brunke, S., Albrecht, A., Thewes, S., Laue, M., Edwards, J. E., Filler, S. G. & Hube, B. (2008). The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog* **4**, e1000217.
- Alsteens, D., Garcia, M. C., Lipke, P. N. & Dufrêne, Y. F. (2010). Force-induced formation and propagation of adhesion nanodomains in living fungal cells. *Proc Natl Acad Sci U S A* **107**, 20744–20749.
- Alvarez, F. J. & Konopka, J. B. (2007). Identification of an *N*-acetylglucosamine transporter that mediates hyphal induction in *Candida albicans*. *Mol Biol Cell* **18**, 965–975.
- Argimón, S., Wishart, J. A., Leng, R., Macaskill, S., Mavor, A., Alexandris, T., Nicholls, S., Knight, A. W., Enjalbert, B. & other authors (2007). Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. *Eukaryot Cell* **6**, 682–692.
- Bailey, D. A., Feldmann, P. J., Bovey, M., Gow, N. A. & Brown, A. J. (1996). The *Candida albicans* *HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J Bacteriol* **178**, 5353–5360.
- Biswas, S., Van Dijk, P. & Datta, A. (2007). Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol Mol Biol Rev* **71**, 348–376.
- Braun, B. R. & Johnson, A. D. (2000). *TUP1*, *CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans*. *Genetics* **155**, 57–67.
- Braun, B. R., Head, W. S., Wang, M. X. & Johnson, A. D. (2000). Identification and characterization of *TUP1*-regulated genes in *Candida albicans*. *Genetics* **156**, 31–44.
- Brena, S., Omaetxebarria, M. J., Elgueababal, N., Cabezas, J., Moragues, M. D. & Pontón, J. (2007). Fungicidal monoclonal antibody C7 binds to *Candida albicans* Als3. *Infect Immun* **75**, 3680–3682.
- Bromuro, C., Torosantucci, A., Gomez, M. J., Urbani, F. & Cassone, A. (1994). Differential release of an immunodominant 65 kDa manno-protein antigen from yeast and mycelial forms of *Candida albicans*. *J Med Vet Mycol* **32**, 447–459.
- Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V. & Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* **37** (Database issue), D233–D238.
- Castilla, R., Passeron, S. & Cantore, M. L. (1998). *N*-acetyl-D-glucosamine induces germination in *Candida albicans* through a mechanism sensitive to inhibitors of cAMP-dependent protein kinase. *Cell Signal* **10**, 713–719.
- Castillo, L., Calvo, E., Martínez, A. I., Ruiz-Herrera, J., Valentín, E., Lopez, J. A. & Sentandreu, R. (2008). A study of the *Candida albicans* cell wall proteome. *Proteomics* **8**, 3871–3881.
- Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T. & Ghannoum, M. A. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* **183**, 5385–5394.
- Coleman, D. A., Oh, S. H., Zhao, X. & Hoyer, L. L. (2010). Heterogeneous distribution of *Candida albicans* cell-surface antigens demonstrated with an Als1-specific monoclonal antibody. *Microbiology* **156**, 3645–3659.
- De Bernardis, F., Mühlischlegel, F. A., Cassone, A. & Fonzi, W. A. (1998). The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect Immun* **66**, 3317–3325.
- de Boer, A. D., de Groot, P. W., Weindl, G., Schaller, M., Riedel, D., Diez-Orejas, R., Klis, F. M., de Koster, C. G., Dekker, H. L. & other authors (2010). The *Candida albicans* cell wall protein Rhd3/Pga29 is abundant in the yeast form and contributes to virulence. *Yeast* **27**, 611–624.
- de Groot, P. W., de Boer, A. D., Cunningham, J., Dekker, H. L., de Jong, L., Hellingwerf, K. J., de Koster, C. & Klis, F. M. (2004). Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot Cell* **3**, 955–965.
- De Groot, P. W., Ram, A. F. & Klis, F. M. (2005). Features and functions of covalently linked proteins in fungal cell walls. *Fungal Genet Biol* **42**, 657–675.
- de Koning, L. J., Kasper, P. T., Back, J. W., Nessen, M. A., Vanrobaeys, F., Van Beeumen, J., Gherardi, E., de Koster, C. G. & de Jong, L. (2006). Computer-assisted mass spectrometric analysis of naturally occurring and artificially introduced cross-links in proteins and protein complexes. *FEBS J* **273**, 281–291.
- Desjardins, P., Hansen, J. B. & Allen, M. (2009). Microvolume spectrophotometric and fluorometric determination of protein concentration. *Curr Protoc Protein Sci Chapter* **3**, 3, 10.
- Ecker, M., Deutzmann, R., Lehle, L., Mersa, V. & Tanner, W. (2006). Pir proteins of *Saccharomyces cerevisiae* are attached to β -1,3-glucan by a new protein-carbohydrate linkage. *J Biol Chem* **281**, 11523–11529.
- Eisenhaber, B., Schneider, G., Wildpaner, M. & Eisenhaber, F. (2004). A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *J Mol Biol* **337**, 243–253.
- Emanuelsson, O., Brunak, S., von Heijne, G. & Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* **2**, 953–971.
- Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F. C. & Hube, B. (2005). Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* **56**, 397–415.
- Frohner, I. E., Bourgeois, C., Yatsyk, K., Majer, O. & Kuchler, K. (2009). *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol Microbiol* **71**, 240–252.

- Gil-Navarro, I., Gil, M. L., Casanova, M., O'Connor, J. E., Martínez, J. P. & Gozalbo, D. (1997). The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is a surface antigen. *J Bacteriol* **179**, 4992–4999.
- Gillum, A. M., Tsay, E. Y. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol Gen Genet* **198**, 179–182.
- Gomez, M. J., Torosantucci, A., Arancia, S., Maras, B., Parisi, L. & Cassone, A. (1996). Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP65), a main target of anti-*Candida* cell-mediated immune responses in humans. *Infect Immun* **64**, 2577–2584.
- Granger, B. L., Flenniken, M. L., Davis, D. A., Mitchell, A. P. & Cutler, J. E. (2005). Yeast wall protein 1 of *Candida albicans*. *Microbiology* **151**, 1631–1644.
- Green, C. B., Cheng, G., Chandra, J., Mukherjee, P., Ghannoum, M. A. & Hoyer, L. L. (2004). RT-PCR detection of *Candida albicans* *ALS* gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. *Microbiology* **150**, 267–275.
- Hayek, P., Dib, L., Yazbeck, P., Beyrouthy, B. & Khalaf, R. A. (2010). Characterization of Hwp2, a *Candida albicans* putative GPI-anchored cell wall protein necessary for invasive growth. *Microbiol Res* **165**, 250–258.
- Heijnis, W. H., Dekker, H. L., de Koning, L. J., Wierenga, P. A., Westphal, A. H., de Koster, C. G., Gruppen, H. & van Berkel, W. J. (2011). Identification of the peroxidase-generated intermolecular dityrosine cross-link in bovine α -lactalbumin. *J Agric Food Chem* **59**, 444–449.
- Hoyer, L. L., Payne, T. L., Bell, M., Myers, A. M. & Scherer, S. (1998). *Candida albicans* *ALS3* and insights into the nature of the *ALS* gene family. *Curr Genet* **33**, 451–459.
- Ibrahim, A. S., Mirbod, F., Filler, S. G., Banno, Y., Cole, G. T., Kitajima, Y., Edwards, J. E., Jr, Nozawa, Y. & Ghannoum, M. A. (1995). Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect Immun* **63**, 1993–1998.
- Kadosh, D. & Johnson, A. D. (2005). Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. *Mol Biol Cell* **16**, 2903–2912.
- Kasper, P. T., Back, J. W., Vitale, M., Hartog, A. F., Roseboom, W., de Koning, L. J., van Maarseveen, J. H., Muijsers, A. O., de Koster, C. G. & de Jong, L. (2007). An aptly positioned azido group in the spacer of a protein cross-linker for facile mapping of lysines in close proximity. *ChemBioChem* **8**, 1281–1292.
- Klis, F. M., Sosinska, G. J., de Groot, P. W. & Brul, S. (2009). Covalently linked cell wall proteins of *Candida albicans* and their role in fitness and virulence. *FEMS Yeast Res* **9**, 1013–1028.
- Klis, F. M., Brul, S. & De Groot, P. W. (2010). Covalently linked wall proteins in ascomycetous fungi. *Yeast* **27**, 489–493.
- Kulkarni, R. D., Kelkar, H. S. & Dean, R. A. (2003). An eight-cysteine-containing CFEM domain unique to a group of fungal membrane proteins. *Trends Biochem Sci* **28**, 118–121.
- Lehmann, E. L. & D'Abrera, H. J. M. (1975). *Nonparametrics: Statistical Methods Based on Ranks*. San Francisco: McGraw-Hill International Book Co.
- Lo, H. J., Köhler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939–949.
- Lorenz, M. C., Bender, J. A. & Fink, G. R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3**, 1076–1087.
- Luo, G., Ibrahim, A. S., Spellberg, B., Nobile, C. J., Mitchell, A. P. & Fu, Y. (2010). *Candida albicans* Hyr1p confers resistance to neutrophil killing and is a potential vaccine target. *J Infect Dis* **201**, 1718–1728.
- Maidan, M. M., Thevelein, J. M. & Van Dijk, P. (2005). Carbon source induced yeast-to-hypha transition in *Candida albicans* is dependent on the presence of amino acids and on the G-protein-coupled receptor Gpr1. *Biochem Soc Trans* **33**, 291–293.
- Martchenko, M., Alarco, A. M., Harcus, D. & Whiteway, M. (2004). Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced *SOD5* gene. *Mol Biol Cell* **15**, 456–467.
- Martínez, A. I., Castillo, L., Garcerá, A., Elorza, M. V., Valentín, E. & Sentandreu, R. (2004). Role of Pir1 in the construction of the *Candida albicans* cell wall. *Microbiology* **150**, 3151–3161.
- Monteoliva, L., Martínez-López, R., Pitarch, A., Hernaez, M. L., Serna, A., Nombela, C., Albar, J. P. & Gil, C. (2011). Quantitative proteome and acidic subproteome profiling of *Candida albicans* yeast-to-hypha transition. *J Proteome Res* **10**, 502–517.
- Müller, M. O., de Koning, L. J., Schmidt, A., Ihling, C., Syha, Y., Rau, O., Mechtler, K., Schubert-Zsilavecz, M. & Sinz, A. (2009). An innovative method to study target protein–drug interactions by mass spectrometry. *J Med Chem* **52**, 2875–2879.
- Naglik, J., Albrecht, A., Bader, O. & Hube, B. (2004). *Candida albicans* proteinases and host/pathogen interactions. *Cell Microbiol* **6**, 915–926.
- Nantel, A., Dignard, D., Bachewich, C., Harcus, D., Marcil, A., Bouin, A. P., Sensen, C. W., Hogues, H., van het Hoog, M. & other authors (2002). Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol Biol Cell* **13**, 3452–3465.
- Nobile, C. J. & Mitchell, A. P. (2005). Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Curr Biol* **15**, 1150–1155.
- Nobile, C. J., Andes, D. R., Nett, J. E., Smith, F. J., Yue, F., Phan, Q. T., Edwards, J. E., Filler, S. G. & Mitchell, A. P. (2006). Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathog* **2**, e63.
- Oda, Y., Huang, K., Cross, F. R., Cowburn, D. & Chait, B. T. (1999). Accurate quantitation of protein expression and site-specific phosphorylation. *Proc Natl Acad Sci U S A* **96**, 6591–6596.
- Otoo, H. N., Lee, K. G., Qiu, W. & Lipke, P. N. (2008). *Candida albicans* Als adhesins have conserved amyloid-forming sequences. *Eukaryot Cell* **7**, 776–782.
- Pérez, A., Pedrós, B., Murgui, A., Casanova, M., López-Ribot, J. L. & Martínez, J. P. (2006). Biofilm formation by *Candida albicans* mutants for genes coding fungal proteins exhibiting the eight-cysteine-containing CFEM domain. *FEMS Yeast Res* **6**, 1074–1084.
- Phan, Q. T., Myers, C. L., Fu, Y., Sheppard, D. C., Yeaman, M. R., Welch, W. H., Ibrahim, A. S., Edwards, J. E., Jr & Filler, S. G. (2007). Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* **5**, e64.
- Popolo, L., Ragni, E., Carotti, C., Palomares, O., Aardema, R., Back, J. W., Dekker, H. L., de Koning, L. J., de Jong, L. & de Koster, C. G. (2008). Disulfide bond structure and domain organization of yeast $\beta(1,3)$ -glucanotransferases involved in cell wall biogenesis. *J Biol Chem* **283**, 18553–18565.
- Ramsook, C. B., Tan, C., Garcia, M. C., Fung, R., Soybelman, G., Henry, R., Litewka, A., O'Meally, S., Otoo, H. N. & other authors (2010). Yeast cell adhesion molecules have functional amyloid-forming sequences. *Eukaryot Cell* **9**, 393–404.
- Richard, M. L. & Plaine, A. (2007). Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*. *Eukaryot Cell* **6**, 119–133.

- Schaller, M., Borelli, C., Korting, H. C. & Hube, B. (2005). Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48**, 365–377.
- Silverman, R. J., Nobbs, A. H., Vickerman, M. M., Barbour, M. E. & Jenkinson, H. F. (2010). Interaction of *Candida albicans* cell wall Als3 protein with *Streptococcus gordonii* SspB adhesin promotes development of mixed-species communities. *Infect Immun* **78**, 4644–4652.
- Simonetti, N., Strippoli, V. & Cassone, A. (1974). Yeast–mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. *Nature* **250**, 344–346.
- Skrzypek, M. S., Arnaud, M. B., Costanzo, M. C., Inglis, D. O., Shah, P., Binkley, G., Miyasato, S. R. & Sherlock, G. (2010). New tools at the *Candida* Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res* **38** (Database issue), D428–D432.
- Sohn, K., Urban, C., Brunner, H. & Rupp, S. (2003). *EFG1* is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. *Mol Microbiol* **47**, 89–102.
- Sorgo, A. G., Heilmann, C. J., Dekker, H. L., Brul, S., de Koster, C. G. & Klis, F. M. (2010). Mass spectrometric analysis of the secretome of *Candida albicans*. *Yeast* **27**, 661–672.
- Sorgo, A. G., Heilmann, C. J., Dekker, H. L., Bekker, M., Brul, S., de Koster, C. G., de Koning, C. J. & Klis, F. M. (2011). The effects of fluconazole on the secretome, the wall proteome and wall integrity of the clinical fungus *Candida albicans*. *Eukaryot Cell* doi:10.1128/EC.05011-11 (in press).
- Sosinska, G. J., de Koning, L. J., de Groot, P. W., Manders, E. M., Dekker, H. L., Hellingwerf, K. J., de Koster, C. G. & Klis, F. M. (2011). Mass spectrometric quantification of the adaptations in the wall proteome of *Candida albicans* in response to ambient pH. *Microbiology* **157**, 136–146.
- Spellberg, B. J., Ibrahim, A. S., Avanesian, V., Fu, Y., Myers, C., Phan, Q. T., Filler, S. G., Yeaman, M. R. & Edwards, J. E., Jr (2006). Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J Infect Dis* **194**, 256–260.
- Staab, J. F., Bradway, S. D., Fidel, P. L. & Sundstrom, P. (1999). Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283**, 1535–1538.
- Theiss, S., Ishdorj, G., Brenot, A., Kretschmar, M., Lan, C. Y., Nichterlein, T., Hacker, J., Nigam, S., Agabian, N. & Köhler, G. A. (2006). Inactivation of the phospholipase B gene *PLB5* in wild-type *Candida albicans* reduces cell-associated phospholipase A2 activity and attenuates virulence. *Int J Med Microbiol* **296**, 405–420.
- Urban, C., Sohn, K., Lottspeich, F., Brunner, H. & Rupp, S. (2003). Identification of cell surface determinants in *Candida albicans* reveals Tsa1p, a protein differentially localized in the cell. *FEBS Lett* **544**, 228–235.
- Weissman, Z. & Kornitzer, D. (2004). A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. *Mol Microbiol* **53**, 1209–1220.
- Weissman, Z., Shemer, R., Conibear, E. & Kornitzer, D. (2008). An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. *Mol Microbiol* **69**, 201–217.
- Wheeler, R. T. & Fink, G. R. (2006). A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog* **2**, e35.
- Wheeler, R. T., Kombe, D., Agarwala, S. D. & Fink, G. R. (2008). Dynamic, morphotype-specific *Candida albicans* β -glucan exposure during infection and drug treatment. *PLoS Pathog* **4**, e1000227.
- Wisplinghoff, H., Bischoff, T., Tallent, S. M., Seifert, H., Wenzel, R. P. & Edmond, M. B. (2004). Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin Infect Dis* **39**, 309–317.
- Xu, X. L., Lee, R. T., Fang, H. M., Wang, Y. M., Li, R., Zou, H., Zhu, Y. & Wang, Y. (2008). Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host Microbe* **4**, 28–39.
- Yin, Q. Y., de Groot, P. W., Dekker, H. L., de Jong, L., Klis, F. M. & de Koster, C. G. (2005). Comprehensive proteomic analysis of *Saccharomyces cerevisiae* cell walls: identification of proteins covalently attached via glycosylphosphatidylinositol remnants or mild alkali-sensitive linkages. *J Biol Chem* **280**, 20894–20901.
- Zhao, X., Oh, S. H., Yeater, K. M. & Hoyer, L. L. (2005). Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151**, 1619–1630.

Edited by: K. Haynes