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Impact of the unfolded protein response upon genome-wide expression patterns, and the role of Hac1 in the polarized growth, of *Candida albicans*

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A B S T R A C T

The unfolded protein response (UPR) regulates the expression of genes involved in the protein secretory pathway and in endoplasmic reticulum (ER) stress in yeasts and filamentous fungi. We have characterized the global transcriptional response of *Candida albicans* to ER stresses (dithiothreitol and tunicamycin) and established the impact of the transcription factor Hac1 upon this response. Expression of *C. albicans* Hac1, which is the functional homologue of *Saccharomyces cerevisiae* Hac1p, is predicted to be translationally regulated via an atypical mRNA splicing event during ER stress. *C. albicans* genes involved in secretion, vesicle trafficking, stress responses and cell wall biogenesis are up-regulated in response to ER stress, and translation and ribosome biogenesis genes are down-regulated. Hac1 is not essential for *C. albicans* viability, but plays a major role in this stress-related transcriptional response and is required for resistance to ER stress. In addition, we show that Hac1 plays an important role in regulating the morphology of *C. albicans* and in the expression of genes encoding cell surface proteins during ER stress, factors that are important in virulence of this fungal pathogen.

Keywords:

HAC1
Pseudohyphae
UPR
Candida albicans
UPR
ER stress

1. Introduction

Candida albicans is a commensal organism in humans that, under some circumstances, causes diseases which range from mucosal colonisation of otherwise healthy people (oral thrush and vaginitis) to invasive infections of people with impaired immune systems (Calderone, 2002). The ability of *C. albicans* to colonize diverse tissues and artificial surfaces such as catheters suggests a phenotypic versatility in its cell surface properties (reviewed by Verstrepen et al., 2004; Verstrepen and Klis, 2006) as well the existence of robust adaptive environmental responses.

Candida albicans can grow as budding yeast, in a pseudohyphal form or as true hyphae depending upon the environmental conditions (Sudbery et al., 2004). Growth is polarized in *C. albicans* hyphae, with continuous apical growth throughout the cell cycle and parallel cell walls at the septal junctions. In contrast, the growth of pseudohyphae and blastophores is only limited to the

apical tip during the initial part of the cell cycle (Court and Sudbery, 2007). *C. albicans* pseudohyphae have a constriction at the septal junction between mother and daughter cells. Pseudohyphae can be distinguished from true hyphae on the basis of their morphological index (Mi) which quantifies the dimensions of cell compartments (Merson-Davis and Odds, 1989). Alternatively they can be distinguished on the basis of the positions of their septal junctions. These lie at the bud neck for pseudohyphae, and within the germ tube for an emerging hypha (Sudbery et al., 2004).

The yeast-hyphal transition is regulated by a complex network of signalling pathways that include evolutionarily conserved MAP kinase and Ras-cAMP modules (reviewed by Ernst, 2000; Gow et al., 2002; Sudbery et al., 2004; Whiteway and Oberholzer, 2004; Brown et al., 2007). The yeast-like form is thought to promote dissemination within the blood stream whereas hyphal development appears to promote invasion (Gow et al., 2002; Sundström, 2006). The link between specific morphotypes and virulence is not firmly established (Gow et al., 2002). However, hyphae are important for the formation of biofilms, which can provide reservoirs of infection in the host, and morphogenetic variation is likely to be important in responding rapidly to environmental change and to host defence mechanisms (Odds, 1988).

Generally, growth of *C. albicans* in the yeast form is favoured at high cell densities, temperatures below 30 °C, and ambient pH below 4. In contrast, the hyphal form is favoured at temperatures

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above 37 °C, at ambient pH close to 7, at lower cell densities, at high CO₂ concentrations, under some limiting nutrient conditions such as amino acid starvation or hypoxia, or in the presence of serum (Odds, 1988; Tripathi et al., 2002; Enjalbert and Whiteway, 2005; Klengel et al., 2005). The effect of serum is complex but it is proposed to act, in part, by conferring amino acid starvation (Tripathi et al., 2002) although other factors are implicated (Maidan et al., 2005; Hudson et al., 2004).

The mechanisms of fungal protein secretion are similar to those in other eukaryotes (Schröder and Kaufman, 2005a,b). The assisted folding of proteins within the lumen of the endoplasmic reticulum (ER) represents a critical step in the secretory pathway because, at this stage, improperly folded proteins are detected and degraded. A build-up of unfolded proteins induces the unfolded protein response (UPR) in the model yeast *Saccharomyces cerevisiae*. This response is triggered by the ER trans-membrane sensor and endonuclease Ire1p, which leads to activation of the transcript encoding the bZIP transcription factor Hac1p (sometimes referred to as Hac1pⁱ where 'i' means induced). Hac1p synthesis is dependent on the splicing of an atypical (non-spliceosomal) intron in the *HAC1* mRNA in all organisms examined to date. The *HAC1* intron is 252 bp in *S. cerevisiae* (Cox and Walter, 1996; Mori et al., 1996) but about 20 bp long in filamentous fungi (Mulder et al., 2004; Saloheimo et al., 2003). This splicing event is activated in response to ER stress leading to the synthesis of functional Hac1p.

The Hac1 protein binds to specific motifs (unfolded protein response elements, UPRs) in responsive promoters to induce the transcription of *S. cerevisiae* genes encoding a variety of ER-resident chaperones and foldases, components of the protein secretory pathway, and genes involved in the ER-associated degradation (ERAD) of improperly-folded proteins. Travers et al. (2000) showed that nearly 400 *S. cerevisiae* genes are induced following exposure to dithiothreitol (DTT) and tunicamycin, which are chemical inducers of the UPR. DTT is thought to induce the UPR by affecting the redox status of the ER lumen, which is important for the formation of disulphide bonds. Tunicamycin is an inhibitor of N-linked glycosylation of secretory proteins. Therefore, while DTT and tunicamycin both induce the UPR, they do not affect the transcription of identical sets of genes (Travers et al., 2000). The UPR has a profound impact upon *S. cerevisiae*, affecting the expression of genes involved in the protein folding, cell surface architecture and secretion (Travers et al., 2000; Kimata et al., 2005).

The roles of the UPR in *S. cerevisiae* have been extended with the realization that the UPR is a key sensor and mediator of nitrogen-regulated effects. Activation of the *HAC1* mRNA by splicing occurs only under nitrogen-sufficient conditions, and down-regulation of the UPR by nitrogen starvation leads to pseudohyphal growth in *S. cerevisiae* (Schröder et al., 2000). The transcriptional activation

of UPR-sensitive genes involves both Hac1p and Gcn4p, two nitrogen-sensitive bZIP transcription factors (Patil et al., 2004). Further complexity in UPR regulation is also apparent (Leber et al., 2004; Schröder et al., 2003), indicating that this response is a versatile and sensitive regulatory mechanism involved in environmental adaptation and cellular development (Kaufman et al., 2002).

The extent of the UPR has not been explored in *C. albicans*. We reasoned that the UPR might be involved in the regulation of polarized growth in this pathogen for two main reasons. Firstly, the UPR is likely to influence the delivery of newly synthesized proteins to the apical tip, which is essential for polarized growth. Secondly, there is interconnection between the Hac1p and Gcn4p regulons in *S. cerevisiae* (Patil et al., 2004), and Gcn4p has been implicated in the regulation of filamentous growth in *C. albicans* (Tripathi et al., 2002; Tournu et al., 2005). Therefore, we have defined the genome-wide transcriptional response of *C. albicans* during the UPR, and confirmed that Hac1 plays an important role in hyphal development.

2. Materials and methods

2.1. *C. albicans* strains, plasmids and growth conditions

The *C. albicans* and *S. cerevisiae* strains used in this study are described in Table 1. Strains were grown in YPD containing 80 µg uridine/ml, or synthetic complete (SC) medium (Sherman, 1991). For low nitrogen culturing conditions, we used SD medium without (NH₄)₂SO₄ and amino acids (Formedium), supplemented with 2% (w/v) glucose (Sherman, 1991). Morphogenesis was stimulated by transferring *C. albicans* yeast cells into RPMI-1640 (containing 25 mM HEPES and NaHCO₃ without L-glutamine) (Sigma) or by adding 20% (v/v) foetal calf serum (Invitrogen) to yeast-like cells growing in YPD or SC at 37 °C (Swoboda et al., 1994). The impact of amino acid starvation on the UPR was tested using the histidine analogue, 3-aminotriazole (3AT), which imposes histidine starvation in *C. albicans* (Tripathi et al., 2002; Tournu et al., 2005). Cells were grown in SC medium lacking histidine, with 30 mM 3 AT at 37 °C for 4 h. To stimulate the UPR in *C. albicans*, stationary phase cells were grown to mid-exponential phase (OD₆₀₀ = 0.5) in YPD at 30 °C with shaking (180 rpm), prior to the addition of 5 mM dithiothreitol (DTT) or 4.73 µM (4 µg/ml) tunicamycin for 1 h. Control cultures were treated with the equivalent volumes of carrier (distilled water and DMSO, respectively).

2.2. Identification and analysis of *C. albicans* *HAC1* sequences

The *C. albicans* *HAC1* locus was identified by interrogating the *C. albicans* genome (<http://genolist.pasteur.fr/CandidaDB>) with

Table 1
C. albicans and *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
<i>C. albicans</i> strains		
SC5314	Prototrophic, clinical isolate	Gillum et al. (1984)
BWP17	ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG	Wilson et al. (1999)
TW01	ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG, hac1::loxP/hac1::loxP	This study
TW02 (WT)	ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG, Clp30 (URA3, HIS1, ARG4)	This study
TW03 (hac1/hac1)	ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG, hac1::loxP/hac1::loxP, Clp30 (URA3, HIS1, ARG4)	This study
TW04 (hac1/hac1/HAC1)	ura3::λimm434/ura3::λimm434, his1::hisG/his1::hisG, arg4::hisG/arg4::hisG, hac1::loxP/hac1::loxP, Clp30-HAC1 (URA3, HIS1, ARG4)	This study
<i>S. cerevisiae</i> strains		
BY4741	MATa his3Δ1leu2Δ0 met15Δ0 ura3Δ0	Euroscarf
BY4741-hac1	MATa his3Δ1leu2Δ0 met15Δ0 ura3Δ0 hac1Δ0	Euroscarf
BY4741-Hac1VTUTW01	MATa his3Δ1leu2Δ0met15Δ0ura3Δ0 hac1Δ0+pVTUTW01	This study
BY4741-Hac1VTUTW02	MATa his3Δ1leu2Δ0met15Δ0ura3Δ0 hac1Δ0+pVTUTW02	This study

S. cerevisiae *HAC1* (YFL031W, <http://db.yeastgenome.org>) using Blastn. Putative DNA-binding domains in the *C. albicans* *HAC1* ORF were identified by Pfam searches <http://pfam.wustl.edu/text-search.shtml>. Multiple-sequence alignments were constructed by using CLUSTAL W 1.6. RNA secondary structure prediction was performed with software from <http://www.bioinfo.rpi.edu/applications/mfold/old/rna/>.

To confirm the atypical intron in the *HAC1* transcript, total RNA was extracted from *C. albicans* cells exposed to DTT or tunicamycin stress using Trizol (Invitrogen) (Hauser et al., 1998). Reverse transcription-polymerase chain reaction (RT-PCR) was performed on these RNA preparations using SuperScript III (Invitrogen) using the primers Hac1IF (5'-TGAGGATGAACACCAAGAAGAA) and Hac1IR (5'-TCAAAGTCCAACCTG AAATGAT). The PCR products were cloned into pGEM[®]-T Easy (Promega) and sequenced on both strands (Sambrook et al., 1989).

To test for the presence of spliced and unspliced *HAC1* transcripts, total RNA was isolated from test *C. albicans* cells (Hauser et al., 1998) and RT-PCR performed using primers that lie either side of the 19 bp intron: HAC1SPF (5'-AGACGCTTTTAATTACCACAC CA) and HAC1IR (5'-TCAAAGTCCAACCTGAAATG). PCR amplification parameters were as follows: 95 °C for 5 min; 30 cycles consisting of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C; and 10 min at 72 °C. The intron-containing *ACT1* sequence was used as a loading control and to test for genomic DNA contamination.

For RACE-PCR experiments, total RNA prepared from *C. albicans* cells was reverse-transcribed using SuperScript III and oligo-dT (20) primer (Invitrogen), and the Marathon cDNA Amplification Kit used following the manufacturer's instructions (Clontech). Amplification of cDNAs corresponding to *HAC1* mRNA 3'-ends was performed using the Marathon cDNA amplification kit with the PCR anchor primer (3'RACE/AP1: Clontech) and the *HAC1*-specific primer, 5'-TGAGGATGAACACCAAGAAGAAGGTCA. Amplification of cDNA corresponding to *HAC1* mRNA 5'-ends was performed using the *HAC1*-specific primer 5'-TGACTTCTTCTGGGT TCACCTA with 5'RACE/AP1 (Clontech) for the first round of PCR. The second rounds of PCR were performed using the primer pairs 5'RACE/AP2 with 5'-TGGCTCTTTCTAGGTGGCAAAGTT (5'-*HAC1*-specific), and 3'RACE/AP2 with 5'-AGACGCTTTTGGAAATACCCATC ACCA (3'-*HAC1*-specific) for the 5' and end 3' RACE analyses, respectively. The PCR products were cloned into pGEM[®]-T Easy and sequenced.

2.3. Strain construction

The *hac1/hac1* null mutant was created using *C. albicans* BWP17 (Table 1) using the *cre-loxP* system described previously (Dennison et al., 2005). Briefly, *hac1::loxP-ARG4-loxP* (*hac1::LAL*) and *hac1::loxP-HIS1-loxP* (*hac1::LHL*) disruption cassettes containing about 120 bp of sequence homology to the 5' and 3' ends of the *HAC1* open reading frame (ORF), were generated by PCR using the primers HACdelF (5'-ATGGAGTAACTGTTGATAACACCAATAC TACTTCCAATATAGATGATTTATCTGTTGCTACACCAACTTCTTTAATGA CAAGTACTACTACATCACCATCAATGTCAACATCAACAAGTCCAGGGTT TTCCAGTCCAG) and HACdelR (5'-ATTATATTCTGGGAGCGCCAAAG AAATTTAGACTTTATGAACCTCAACATCATCTCTAAATCGAAAAAAA ATCATCAAAGTCCAACCTGAAATGATTCTATTGGCTCTGCTGGCTAGCAC TAAAGGGAACAAAAGC) with the plasmid templates pLAL2 or pLHL, respectively (Dennison et al., 2005). The *hac1::LAL* and *hac1::LHL* disruption cassettes delete from +120 to +1032 of the 1074 bp *HAC1* ORF. These cassettes were transformed sequentially into *C. albicans* BWP17 to disrupt both alleles of the *HAC1* gene and generate a *HAC1*⁻, *ARG*⁺, *HIS*⁺, *URI*⁻ mutant. An *arg4::MET3-cre-URA3* cassette was transformed into this mutant and the *cre* resolvase activated on medium lacking methionine/cysteine (Dennison et al., 2005). This generated the *HAC1*⁻, *ARG*⁻, *HIS*⁻, *URI*⁻ mutant,

TWO1 (*hac1::loxP/hac1::loxP*: Table 1), the genotype of which was confirmed by PCR and Southern hybridization.

To reintroduce *HAC1* into *C. albicans* TWO1, the *HAC1* locus was PCR amplified using the primers HAC1SACIF (5'-GAGAGCTCCAAT TTTCGTGCCGTAGG) and HAC1SACIIR (5'-GACCGCGGTTTAGTGGTG GCTGATTTTT) (SacI and SacII sites underlined) and cloned into the plasmid Clp30 (Dennison et al., 2005) to create Clp30-*HAC1* (*hac1*). Clp30, which is a derivative of Clp10 (Murad et al., 2000), contains the *URA3 HIS1* and *ARG4* markers. Clp30-*HAC1* was linearized with *StuI*, transformed into *C. albicans* to create the *HAC1*-reintegrant strain TWO4 (*hac1/hac1/HAC1* Table 1). The integration of Clp30-*HAC1* at the *RPS1* locus in TWO4 was confirmed by PCR using the primers 5'-CACCACTATCATAACCAAGAG and 5'-ATTGTAAATGG GGAATCATGG. The control strains, TWO2 and TWO3, were generated by transforming the parental strain (BWP17) and the *hac1/hac1* mutant (TWO1) with the empty Clp30 plasmid (Table 1), respectively.

2.4. Microscopy

The morphologies of *C. albicans* cells were examined using light and fluorescence microscopy. Cells were grown overnight in YPD, 30 °C with shaking, diluted to OD₆₀₀ = 0.5 in fresh YPD medium with 20% (v/v) foetal calf serum. Cultures were incubated at 37 °C and cells visualised by light microscopy every hour. To analyse the effect of temperature and pH shift, single colonies were isolated and used to inoculate the Soll's medium (pH 4.5) (Buffo et al., 1984). Cultures were grown overnight in Soll's medium, 25 °C with shaking. Five millilitres of this culture was used to inoculate 50 ml Soll's medium (pH 6.5) prewarmed to 37 °C for 5 h. To differentiate the true hyphae from pseudohyphae, *C. albicans* cells were stained with 0.1% (w/v) Calcofluor white to visualize septa through their relatively high chitin content. Cell morphology was analysed using a Zeiss imaging microscope mounted with a 35 mm Nikon Axiocam camera. The morphology index (Mi) of *C. albicans* cells was determined from their maximum length (*l*), maximum diameter (*d*) and septal diameter (*s*) in the ratio (*l*:*s*)/*d*², as described by Merson-Davis and Odds (1989).

2.5. Stress sensitivity

The sensitivities of *C. albicans* strains to DTT, tunicamycin, Calcofluor white, and Congo red (Sigma) were assayed by spotting 10-fold serial dilutions of freshly grown mid-exponential cells onto YPD agar containing these agents at the specified range of concentrations. Growth was monitored after 48 h at 30 °C.

2.6. Complementation tests

We tested the ability of various *C. albicans* *HAC1* cDNA species to complement *S. cerevisiae* *hac1* phenotypes using the strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and the *S. cerevisiae* *hac1* mutant derived from BY4741 (EUROSCARF, Frankfurt, Germany) (Table 1). The expression vector pVTU260 (Melcher, 2000: EUROSCARF) was used to express *C. albicans* *HAC1* cDNAs in *S. cerevisiae*. cDNAs containing unspliced and spliced versions of Ca*HAC1* were amplified by PCR using the primers Ca*HAC1*NHEIF (5'-GAGCT AGCATGGAGTAACTGTTG) and Ca*HAC1*NCOIR (5'-GACCATGGCCG CCAAACA) to create restriction sites (underlined), just before and after the start and stop codons, respectively. The resulting PCR products (1217 bp and 1198 bp) were digested with NheI and NcoI and cloned into pVTU260 between the *ADH1* promoter and terminator sequences to create pVTUTW01 (unspliced *HAC1*) and pVTUTW02 (spliced *HAC1*), which were verified by DNA sequencing. pVTU260, pVTUTW01 and pVTUTW02 were transformed into *S. cerevisiae* (Gietz and Woods, 2002). The

transformants were confirmed by PCR using the primers, 5'-CTCGT TCCCTTCTCCTTG and 5'-GAACCAACTTAT, and their sensitivities to the UPR stresses, DTT and tunicamycin, tested as described above.

2.7. Transcription profiling

Transcript profiling was performed on the *C. albicans* strains BWP17 (*HAC1/HAC1*) and TW01 (*hac1/hac1*) as described previously (Enjalbert et al., 2006). The strains were cultured to $OD_{600} = 0.5$ in YPD supplemented with uridine at 30 °C, at 200 rpm, and then divided into two parts: one for ER stress and one control (carrier). Cells were exposed to DTT (5 mM) or tunicamycin [4 µg/ml (4.73 µM)] for 1 h, collected by centrifugation, snap frozen in liquid N₂, disrupted using a microdismembrator (Braun, Melsungen, Germany) and RNA prepared as described previously (Hauser et al., 1998). To analyse the effect of *HAC1* deletion, *C. albicans* strains BWP17 (*HAC1/HAC1*) and TW01 (*hac1/hac1*) were cultured to $OD_{600} = 0.5$ in YPD supplemented with uridine at 30 °C, at 200 rpm, collected by centrifugation, snap frozen in liquid N₂, disrupted using a microdismembrator and RNA prepared as described above. Cy3- and Cy5-labeled cDNAs were prepared from total RNA, and the probes were hybridized with whole genome microarrays containing ca. 6000 *C. albicans* genes (Eurogentec, Seraing, Belgium). Slides were scanned using a ScanArray Lite scanner (Perkin-Elmer Life Sciences, Beaconsfield, United Kingdom) and quantified using QuantArray software (version 2.0). Preliminary normalization and logarithmic transformation were performed with Excel 2000 (Microsoft). Data normalization and analysis were performed using GeneSpring (Silicon Genetics, Redwood City, CA), and statistical analysis was performed using SAM (Significance Analysis of Microarrays; Tusher et al., 2001). Expression ratios were calculated by comparing stressed cells with their unstressed control in wild-type and mutant strains independently. For *HAC1* deletion analysis, expression ratios were calculated by comparing the TW01 cells with wild-type (BWP17) cells. Data from at least three independent biological replicates were used for each analysis. The data are available at www.ebi.ac.uk/arrayexpress (E-MEXP-1307).

Genes up-regulated due to DTT and tunicamycin treatments (≥ 1.5 -fold) were considered as putative ER stress-induced genes. Genes down-regulated due to *HAC1* deletion (>1.5 -fold) were considered as *Hac1*-dependent genes without ER stress.

3. Results

3.1. *C. albicans* *HAC1*

A presumptive *HAC1* locus (CA0457) was identified in the *C. albicans* genome sequence (<http://genolist.pasteur.fr/CandidaDB/>) via homology to the *S. cerevisiae* *HAC1* gene (YFL031w). The translated sequence of this putative *HAC1* locus displays strong sequence similarity to other fungal *Hac1* protein sequences in the DNA-binding region, but the sequence similarity is limited outside this region (Fig. 1A). Similar observations have been made when comparing other transcription factors in *C. albicans* and *S. cerevisiae*. These functional orthologues often display limited sequence similarity outside their DNA-binding domains (Fig. 1A) (e.g. Stoldt et al., 1997; Tripathi et al., 2002; Martchenko et al., 2007).

During the UPR in other fungal systems *HAC1* mRNA is activated via an atypical splicing event (Cox and Walter, 1996; Mori et al., 1996; Saloheimo et al., 2003; Mulder et al., 2004). Therefore, to test whether CA0457 represents the *C. albicans* *HAC1* gene, we examined the splicing of the CA0457 transcript in response to the ER stresses, DTT or tunicamycin. RT-PCR was performed across the putative *HAC1* mRNA intron using cDNA prepared from ER stressed

and unstressed cells. Splicing of the intron following ER stress was confirmed by DNA sequencing of the shorter RT-PCR products and the intron was shown to be 19 bp (Fig. 1B). This splicing event alters the C-terminal amino acid sequence of the *Hac1* protein. DNA sequencing also confirmed that the *C. albicans* *HAC1* gene was devoid of CUG codons. Furthermore, we identified that the sequence of *HAC1* is incomplete and the full *HAC1* genome sequence data have been submitted to the GenBank database under Accession No. EF655649. Intron borders and splice sites of the *S. cerevisiae* *HAC1* and filamentous fungal (*Aspergillus niger*) *hacA* are well conserved (Calfon et al., 2002), and these conserved bases were also found at the borders of the *C. albicans* *HAC1* (Fig. 1C). We examined the *HAC1* mRNA splicing event in yeast and hyphal cells. There was a clear increase in the abundance of a shorter RT-PCR product following ER stress in yeast cells, consistent with the splicing of the intron from this region of the transcript (Fig. 2A). *HAC1* mRNA splicing was only detected in hyphae following exposure to ER stress (Fig. 2B).

We asked whether *HAC1* mRNA is spliced following amino acid starvation in *C. albicans*. No detectable splicing of the 19 bp *HAC1* mRNA intron was observed in cells treated with 3AT which imposes histidine starvation and activates general amino acid control in *C. albicans* (Tripathi et al., 2002) although splicing was evident in the presence of DTT (Fig. 2B). Furthermore, *HAC1* mRNA splicing was not enhanced further by simultaneous exposure of *C. albicans* cells to DTT and 3AT (Fig. 2B). Note that the effects of exposure to tunicamycin were similar to those of DTT (data not shown).

We performed complementation tests to determine whether CA0457 is a functional homologue of *S. cerevisiae* *HAC1*. cDNAs containing the unspliced (*HAC1^u*) and spliced (*HAC1^s*) versions of *C. albicans* *HAC1* were cloned into the *S. cerevisiae* *ADH1* expression vector, pVTU260 to generate pVTUTW01 and pVTUTW02, respectively. These plasmids were transformed into an *S. cerevisiae* *hac1* null mutant strain and these transformants tested for their sensitivity to DTT and tunicamycin (Fig. 3). As expected, the *S. cerevisiae* *hac1* strain was hypersensitive to DTT and tunicamycin compared with the isogenic wild-type parental strain. This phenotype was partially suppressed by expression of spliced *C. albicans* *HAC1* sequence (pVTUTW02) into *S. cerevisiae* *hac1* cells, but not by the unspliced *C. albicans* *HAC1* cDNA (pVTUTW01). We conclude that *C. albicans* *HAC1* is a functional homologue of *S. cerevisiae* *HAC1*, but that *S. cerevisiae* is probably unable to process accurately the non-spliceosomal intron in the *C. albicans* *HAC1* gene.

3.2. *C. albicans* *HAC1* UTR

In filamentous fungi, ER stress induces truncation of the *HAC1* mRNA in the 5'-untranslated region as well as excision of the non-conventional intron, but this 5' truncation is not observed in *S. cerevisiae* (Rüeggsegger et al., 2001; Mulder et al., 2004). We examined this in *C. albicans* by RACE-PCR with RNA isolated from *C. albicans* cells treated with DTT and from untreated control cells. Three *HAC1* transcriptional start sites were identified in DTT-treated cells, whereas only one start site was detected in unstressed cells (Fig. 4). The unstressed cells produced a longer mRNA, with only one transcription starting point compared to three different start positions in DTT stressed cells which are 41, 74 (41 + 33), and 92 (74 + 18) nucleotides downstream of the non-stressed transcriptional start site. The extended 5'-untranslated region observed in unstressed cells is predicted to form more stable RNA secondary structures ($\Delta G = -16.9$ kcal/mol) than the shorter 5'-untranslated regions in ER stressed cells ($\Delta G = 2.1$ kcal/mol). Therefore *HAC1* transcripts in ER stressed cells have truncated 5'-untranslated regions.

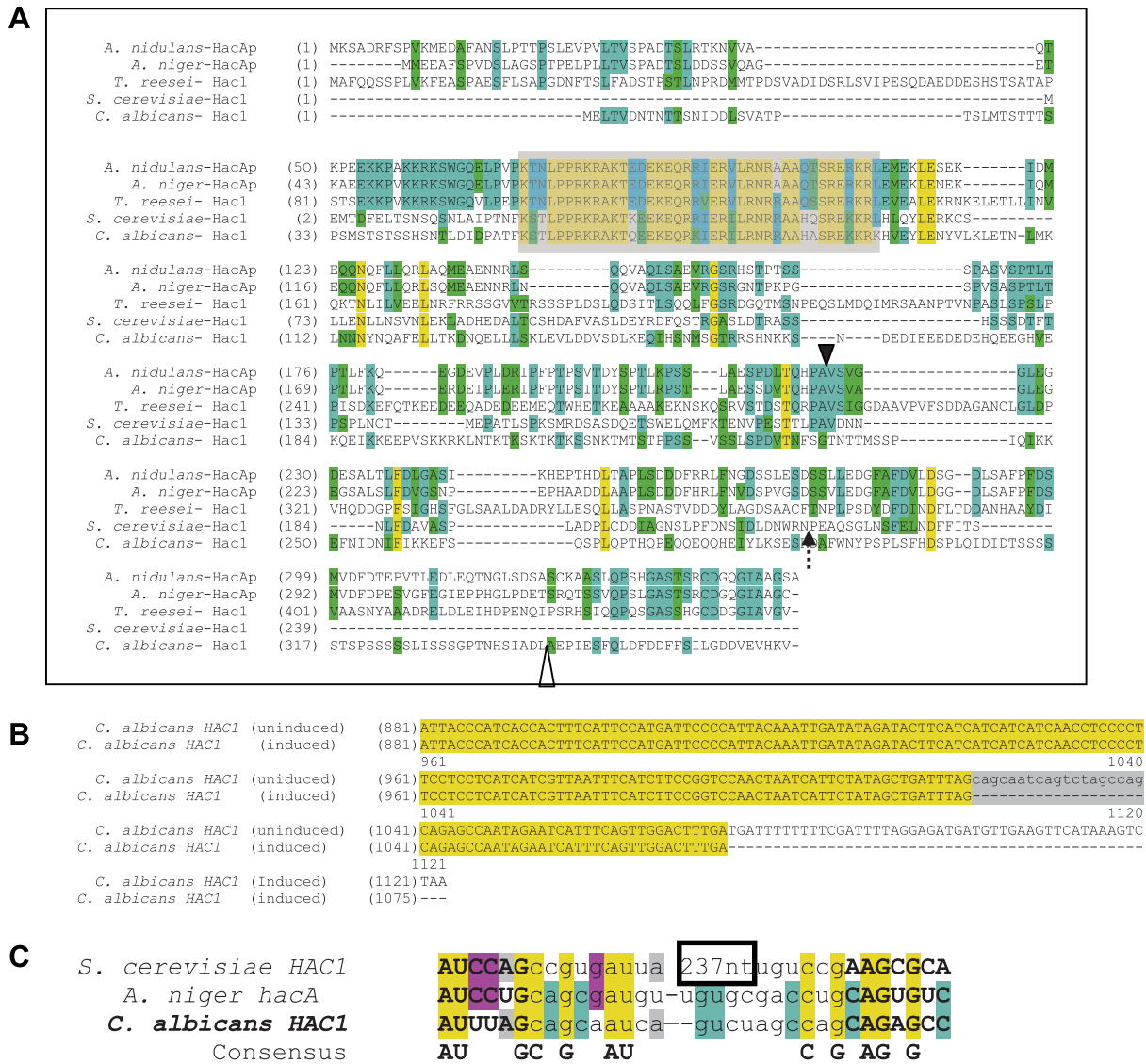


Fig. 1. (A) Alignment of the Hac1 protein sequences from *Aspergillus nidulans*, *A. niger*, *S. cerevisiae*, *T. reesei* and *C. albicans*. Note that *C. albicans* Hac1 has similarity to the other proteins only in the DNA-binding domain area (shaded). The positions of the atypical intron in *A. nidulans*, *A. niger* and *T. reesei* (▼) *S. cerevisiae* (♣) and *C. albicans* (▲) sequences are indicated. (B) DNA sequence alignment of the *C. albicans HAC1* (induced) and *HAC1* (uninduced) gene. The 19bp atypical intron is indicated in lower case. (C) Alignment of the mRNA sequence surrounding the intron in *S. cerevisiae HAC1*, *A. niger hacA* and *C. albicans HAC1*. Intron flanks are indicated by upper case. 237 nt refers to 237 nucleotides out of the 252 nt present in the *S. cerevisiae HAC1* intron.

3.3. *C. albicans HAC1* and ER stress resistance

We generated a homozygous *C. albicans hac1/hac1* null mutant to test whether Hac1 is required for ER stress resistance. Both *HAC1* alleles in this diploid fungus were sequentially deleted, removing nucleotides +121 to +1032 of the 1074 bp ORF. The *ARG4* and *HIS1* markers used in this process were removed by site-specific recombination via the *Cre-loxP* system (Dennison et al., 2005). This allowed the generation of a well-controlled isogenic set of wild-type (*HAC1/HAC1*: TW02), mutant (*hac1/hac1*: TW03) and reintegrated (*hac1/hac1/HAC1*: TW04) strains in which the *ARG4*, *HIS1* and *URA3* markers are all located at the same genomic location (Table 1). In this way we avoided the complication that marker position effects may influence some phenotypes in *C. albicans* (Lay et al., 1998; Sundström et al., 2002; Brand et al., 2004).

The behaviours of these wild-type, mutant and reintegrated strains were compared following ER stress on YPD agar plates. As expected (Chaffin, 1985), wild-type *C. albicans* cells were sensitive to tunicamycin. We observed that Hac1 inactivation reproducibly

increased the sensitivity of *C. albicans* cells to tunicamycin and DTT on plates (Fig. 5) and in liquid culture (not shown). These phenotypes were suppressed by the reintegration of a wild-type *HAC1* gene, confirming that Hac1 is required for ER stress resistance in *C. albicans*. This is consistent with the situation in *S. cerevisiae* (Schröder and Kaufman, 2005a).

3.4. The genome-wide impact of Hac1 in *C. albicans*

The genome-wide role of Hac1 in *C. albicans* was examined by transcript profiling. We compared the genome-wide expression profiles of wild-type and *hac1/hac1* yeast cells in following exposure to the ER stresses, DTT and tunicamycin (Supplementary data Part A). At least three independent replicates were performed for each experimental condition and genes that displayed statistically significant changes in expression level (≥ 1.5 -fold Supplementary data Part B) were identified using SAM (Materials and Methods). Changes in transcript levels were verified by quantitative real-time PCR for six genes under stress conditions (*SEC61:CA3197*), *ALS12*

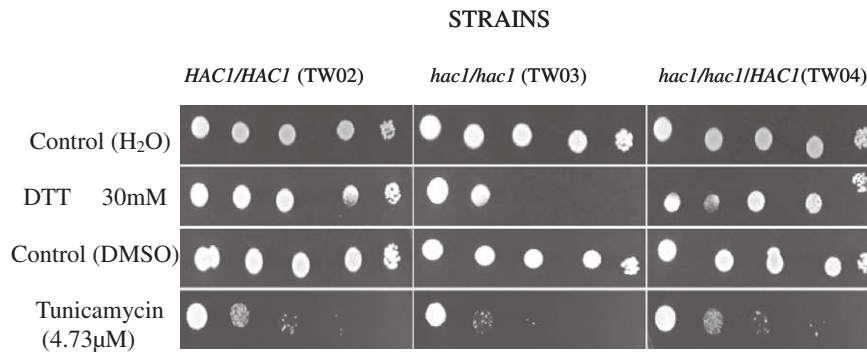


Fig. 5. Sensitivity of *C. albicans* *hac1/hac1* cells to ER stress. Growth of *C. albicans* strains *HAC1/HAC1* (TW02), *hac1/hac1* (TW03) and *hac1/hac1/HAC1* (TW04) on YPD agar medium containing 30 mM DTT or 4.73 μM tunicamycin (4 μg/ml).

during ER stress in *C. albicans*. These included *HAC1* itself, this transcript being 2-fold and 2.9-fold up-regulated in response to DTT and tunicamycin, respectively (Supplementary data, Part B). Other *C. albicans* genes were regulated specifically by DTT or tunicamycin.

We analysed the functional categories that displayed statistically significant enrichment in these subsets of genes compared with the genome as whole (Supplementary data, Part D). Significantly, functional categories relating to protein secretion and cell wall biosynthesis were up-regulated in wild-type *C. albicans* cells following exposure to DTT and tunicamycin. Clearly, Hac1 contributes to this regulation, because much of this regulation was lost or reduced in *hac1/hac1* cells, particularly for DTT-treated cells.

These observations were reinforced by a closer examination of the behaviour of Hac1-dependent genes under these stress conditions. 252 genes were identified as up-regulated (≥ 1.5 -fold differential regulation) in response to both DTT and tunicamycin and were Hac1-dependent (at least 1.5-fold expression ratios of WT/*hac1* in either condition) (Supplementary Data Part E). Within this list, *C. albicans* genes that showed ≥ 1.5 -fold differential regulation in response to both DTT and tunicamycin and were Hac1-dependent (at least 1.5-fold expression ratios of WT/*hac1* in both conditions) in their transcription were listed according to functional category (Fig. 6). These genes were defined as genes regulated by the UPR. Seventy-nine genes are secretion-related and up-regulated in a Hac1-dependent manner in *C. albicans* under stress conditions. One hundred and three genes were similarly up-regulated in *S. cerevisiae* (Travers et al., 2000). Only 12 genes identified as Hac1-regulated in *C. albicans* were also found to be Hac1p-regulated in *S. cerevisiae*; these are indicated in Fig. 6. Furthermore, there were seven genes identified as having no clear homologues in the *S. cerevisiae* genome database. Down-regulated genes are catalogued in Supplementary Data Part F.

Transcriptional profiles of WT and *hac1/hac1* cells were also compared when cells were grown in yeast form in rich (YPD) medium in the absence of either DTT or tunicamycin. From the array data, 126 genes were transcriptionally-dependent on *HAC1* at >1.5 -fold and 66 of those genes had homologues in *S. cerevisiae*. Of those genes, the following have functionality in the secretory process (*CHC1* (CA0801), *COPI* (CA0606), *MYO2* (CA5247), *PEX14* (CA0573), *SEC14* (CA5398), *SEC27* (CA2033), *SRP54* (CA5440), *SRP72* (CA2519) (Supplementary data Parts G). None of the *ALS* genes that are regulated by Hac1 under stress conditions were identified under non-stress conditions.

3.5. *C. albicans* *HAC1*, the cell wall and morphogenesis

Transcript profiling studies suggested that Hac1 influences the transcription of genes, at least under stress conditions, involved in cell wall biogenesis and in secretory and vesicle trafficking pro-

cesses. We reasoned therefore, that *C. albicans* cells might display phenotypes associated with cell wall defects. We tested this by exposing wild-type (TW02), *hac1/hac1* mutant (TW03) and *HAC1*-reintegrant (TW04) strains to cell wall-perturbing agents. When these strains were plated on YPD agar containing Congo red (250 μg/ml) and Calcofluor white (30 μg/ml) (Bates et al., 2005), it was evident that *C. albicans* *hac1/hac1* cells are more sensitive to Congo red and Calcofluor white than the isogenic control strains (Fig. 7). Wild-type and reintegrant strains were sensitive to these cell wall-perturbing agents at higher concentrations compared to the *hac1/hac1* mutant strain (data not shown). This is consistent with the idea suggested by our microarray analyses that Hac1 influences the synthesis of cell wall components.

The morphologies of wild-type (TW02), *hac1/hac1* mutant (TW03) and *HAC1*-reintegrant (TW04) cells were examined following serum-stimulation (Fig. 8). The *hac1/hac1* mutant displayed significant morphological defects that were suppressed by the reintroduction of *HAC1* (Fig. 8A and B). The morphologies of mutant and control cells were quantified using the morphological index (Merson-Davis and Odds, 1989). Hyphal development was reduced from about 80% to 14% in the *hac1/hac1* mutant, with a concomitant increase in pseudohyphal and budding cells under these conditions (Fig. 8C). As shown in Fig. 8D, Growth at 37 °C and near to neutral pH highly favoured pseudohyphae formation in all three strains (Sudbery, 2001). Nevertheless, the formation of hyphae in *hac1/hac1* cells was reduced by 23% compared to wild-type and reintegration strains (Fig. 8D). This confirmed an impact of Hac1 on *C. albicans* morphology, revealing a general reduction in polarized growth in *hac1/hac1* cells.

We also noted that filamentous *hac1/hac1* cells formed smaller and less sticky flocks compared with their isogenic controls (Fig. 8E), suggesting that Hac1 inactivation affected adhesion function at the cell surface. This was consistent with our observation that the *C. albicans* genes that are up-regulated in a Hac1-dependent manner during UPR stress include several adhesins (*ALS4* [CA1528], *ALS5* [CA2852], *ALS12* [*ALS2*:CA0413], *ALS10* [*ALS3*:CA0448]: Hoyer, 2001) and protein mannosyltransferases that are known to affect the functionality of *C. albicans* adhesins (*PMT1*, *PMT2*, *PMT4-6*: Timpel et al., 1998, 2000). Strain comparisons in the absence of ER stress were only possible for yeast cells, and expression of the adhesion genes was not identified as being Hac1-dependent.

4. Discussion

We have shown that *C. albicans* *HAC1* mRNA carries a non-conventional intron whose length (19 bp) is similar to that observed in filamentous fungi and therefore distinct from the 250 bp intron in *HAC1* from *S. cerevisiae*. This *C. albicans* *HAC1* mRNA intron is removed in response to ER stress, as might have been expected based

	1	2	3	4	5	6	
	WT	WT	<i>hac1</i>	<i>hac1</i>	ratio of WT/	ratio of WT/	(<i>hac1/hac1</i>)
	DTT	Tun	DTT	Tun	DTT	Tun	Common name
Translocation							
CA3197*	3.1	3.8	1.1	0.8	2.8	4.9	<i>SEC61</i>
CA1267	2.7	4.7	1.3	1.1	2.0	4.4	<i>YSY6</i>
CA2425*	2.2	2.8	0.8	1.1	2.7	2.5	<i>SEC12</i>
CA5452	1.7	3.3	1.1	1.6	1.6	2.1	<i>SEC11</i>
CA0958*	1.8	2.3	1.3	1.0	1.5	2.4	<i>SEC24</i>
Glycosylation/ Modification							
CA4424*	1.7	2.2	0.7	0.5	2.6	4.1	<i>PMT1</i>
CA4314	2.6	3.0	0.9	0.9	3.0	3.3	<i>PMT4</i>
CA0503	1.6	1.5	0.6	0.7	2.5	2.2	<i>PMT6</i>
CA5003*	2.8	2.4	1.0	1.2	2.7	2.1	<i>MCD4</i>
CA4528	1.6	2.2	1.0	1.1	1.6	2.0	<i>STT3</i>
CA2612*	3.1	3.5	1.3	1.9	2.3	1.8	<i>GAA1</i>
CA2980	2.1	1.8	1.0	1.2	2.1	1.5	<i>GOG5</i>
CA5610	1.8	1.6	0.8	0.9	2.1	1.9	<i>PMR1</i>
CA5144	2.4	1.6	0.9	1.0	2.6	1.5	IPF1019
CA1146	3.6	1.8	1.0	0.9	3.5	2.0	<i>CWH8</i>
CA5894*	1.7	1.5	0.4	0.4	4.2	3.9	<i>PMT2</i>
Protein Folding							
CA0457	2.0	2.9	1.0	1.0	1.9	3.0	<i>HAC1</i>
CA1583	2.3	1.9	1.0	1.4	2.3	1.4	<i>ROT1</i>
Protein degradation							
CA0549	1.5	1.7	1.0	1.1	1.5	1.6	<i>SAP98</i>
CA5322	5.1	2.8	1.5	1.6	3.5	1.8	<i>PRB1</i>
CA0881	2.0	2.7	1.2	1.8	1.6	1.5	IPF16965
CA5735	1.7	1.9	0.9	1.2	1.8	1.5	IPF2878
CA4105	3.6	2.8	2.2	1.9	1.6	1.5	<i>SMT3</i>
Vesicle trafficking/ Transport							
CA2353	1.8	1.6	1.2	0.6	1.5	2.6	<i>VPS13</i>
CA1896*	2.5	3.9	1.5	2.3	1.7	1.7	<i>ERD2</i>
CA4739	2.8	3.2	1.1	1.6	2.5	1.9	IPF4498
CA3230	1.5	1.7	1.0	1.1	1.5	1.5	<i>EMP24</i>
Lipid/Inositol metabolism							
CA3211	3.2	5.5	1.5	1.5	2.2	3.6	IPF19977
CA5986	8.6	2.2	2.5	0.9	3.4	2.4	<i>INO1</i>
CA1956	4.2	1.6	1.7	1.0	2.5	1.7	<i>ERG3</i>
CA3590	3.0	1.7	1.7	1.0	1.8	1.7	<i>ARE2</i>
CA0863	4.2	1.8	2.3	1.1	1.9	1.6	<i>ITR2</i>
CA2934	2.0	1.7	1.2	1.1	1.7	1.5	IPF11515
Cell wall associated proteins							
CA0413	3.5	2.3	0.9	0.8	3.8	2.8	<i>ALS2</i>
CA1528	3.5	2.1	0.9	0.8	3.8	2.7	<i>ALS4</i>
CA2852	1.8	1.6	1.1	0.9	1.6	1.8	<i>ALS5</i>
CA0448	2.0	2.3	1.4	1.3	1.5	1.7	<i>ALS3</i>
CA0882	3.1	2.1	1.5	1.3	2.0	1.7	<i>PHR3</i>
CA0356*	1.8	3.7	1.0	1.2	1.7	3.0	IPF2532
CA5468	1.7	1.7	1.1	1.1	1.6	1.5	<i>IFF7</i>
CA3613*	2.1	1.7	1.0	1.0	2.0	1.8	<i>SPF1</i>
CA2714	7.2	4.0	2.4	1.9	3.0	2.1	<i>IFF2</i>

Fig. 6. Impact of Hac1 inactivation upon the regulation of *C. albicans* genes during ER stress. *C. albicans* genes showing differential regulation in response to DTT and tunicamycin and having homologues in *S. cerevisiae* were clustered according to functional category. Columns (1–4) from left to right correspond to: (1) DTT-treated versus untreated wild-type cells (BWP17); (2) tunicamycin-treated (Tun) versus untreated wild-type cells; (3) DTT-treated versus untreated *hac1/hac1* (TW01) cells; (4) tunicamycin-treated versus untreated *hac1/hac1* (TW01) cells. Two scale bars are presented at the bottom of the figure. The influence of Hac1 on these expression patterns was estimated by dividing the fold regulation for a particular gene under a particular condition in *hac1/hac1* (TW01) cells by the corresponding fold-regulation in wild-type cells (columns 5 and 6). Expression ratios are indicated by the upper of the two scale bars (red, up-regulation; green, down-regulation). The impact of Hac1 upon this regulation was assessed by dividing the fold regulation for a specific gene in wild-type cells by the corresponding regulation in *hac1/hac1* cells; (5) DTT stress; (6) tunicamycin stress. Those genes whose transcription was affected more than 1.5-fold by DTT or tunicamycin and had a ratio of ≥ 1.5 -fold expression change (WT vs Δ hac1) were defined as genes regulated by the UPR in a Hac1-dependent manner. Of these genes, 12 were identified in *S. cerevisiae* (Travers et al., 2000) as being Hac1-dependent UPR genes: they are indicated by an asterisk. Genes identified as having no clear homologues in *S. cerevisiae* are highlighted in bold in the right panel.

Cellular transport							
CA3895	4.9	1.7	2.8	0.5	1.7	3.6	<i>CDR4</i>
CA0609	2.1	3.2	1.2	1.4	1.8	2.3	<i>CDR11</i>
CA3154	4.4	1.9	1.7	1.2	2.6	1.6	<i>FUN34</i>
CA2792*	1.6	1.5	0.9	1.0	1.8	1.5	<i>TRK1.3f</i>
CA0954	1.9	3.2	1.2	2.2	1.6	1.5	IPF13162
CA4669	3.1	3.1	2.0	2.1	1.6	1.5	<i>AVT7</i>
CA0981	2.0	2.0	1.2	1.4	1.6	1.5	<i>GAP4</i>
CA2435	2.8	1.8	1.1	1.2	2.6	1.5	IPF9483
CA4204	1.9	1.9	1.0	1.3	1.8	1.5	<i>HOL4</i>
CA3125*	6.2	2.7	2.1	1.3	3.0	2.1	<i>FCY21.5f</i>
CA3249	2.5	2.2	1.6	1.4	1.6	1.6	<i>GEF2.3f</i>
CA2932	2.0	2.0	1.1	1.3	1.8	1.6	<i>NHX1</i>
CA3545	1.8	1.5	1.2	0.8	1.5	1.8	<i>BPT1</i>
CA5160	1.5	1.8	1.0	1.0	1.5	1.7	<i>PHO89</i>
Other							
CA1010	1.6	2.0	0.9	0.7	1.7	2.7	<i>GLT1</i>
CA2650	2.5	3.6	1.4	1.6	1.8	2.3	<i>VPH1</i>
CA2277	1.9	2.6	1.3	1.2	1.5	2.2	<i>DRS21</i>
CA5890	3.9	2.1	1.1	1.2	3.5	1.7	<i>FCR1</i>
CA6070	2.9	2.1	1.4	1.3	2.1	1.7	<i>RHB1</i>
CA5493	3.4	1.9	1.5	1.2	2.3	1.6	<i>GCN1.5f</i>
CA1288	2.8	2.3	1.3	1.5	2.1	1.5	<i>KSP1</i>
CA2342	2.1	2.0	1.2	1.4	1.7	1.5	ASR1
Unknown function							
CA5868	2.6	2.2	1.5	1.1	1.8	2.1	IPF351
CA5246	1.8	1.8	1.2	0.9	1.5	2.0	IPF19808
CA4951	2.2	3.1	1.5	1.6	1.5	1.9	IPF13607
CA3173	3.5	3.0	1.9	1.6	1.9	1.9	PGA47
CA1476	2.4	2.2	0.9	1.2	2.7	1.8	<i>FLO8</i>
CA5812	3.1	2.7	1.7	1.7	1.8	1.7	
CA6053	2.2	1.5	1.0	0.9	2.2	1.6	IPF4949
CA2874	1.6	2.5	0.7	1.5	2.3	1.6	IPF4999
CA3966	1.9	1.6	1.2	1.1	1.5	1.5	IPF9376
CA1760	2.8	2.6	1.6	1.8	1.8	1.5	<i>RBR3</i>
CA1433	3.4	5.1	2.0	1.6	1.7	3.2	IPF12464
CA3565	1.7	2.1	1.0	1.2	1.8	1.8	<i>MSC2</i>
CA0110	2.2	1.9	0.8	1.1	2.8	1.7	IPF17190
CA4936	8.7	3.2	2.9	1.9	3.0	1.7	IPF8369
CA2405	4.7	2.6	1.9	1.6	2.4	1.6	PGA18

over 1.5	up to 3	up to 5	over 5.1	Induction / repression
over 1.5	up to 3	up to 5	over 5.1	
				Wild-type/TW01 ratio

Fig. 6 (continued)

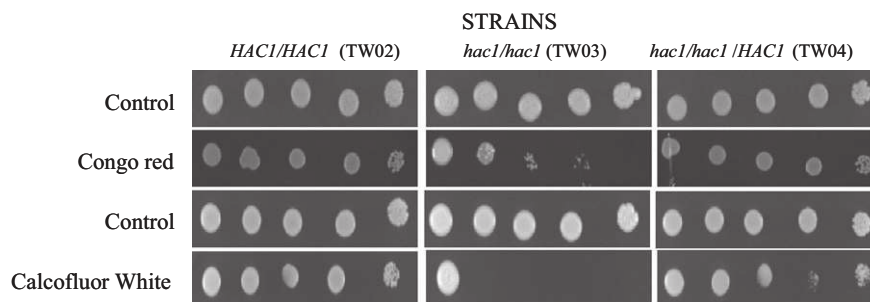


Fig. 7. Sensitivity of the *C. albicans* WT (TW02), *hac1/hac1* (TW03) and *hac1/hac1/HAC1* (TW04) strains to cell wall-perturbing agents, Congo red (250 µg/ml) and Calcofluor white 30 µg/ml.

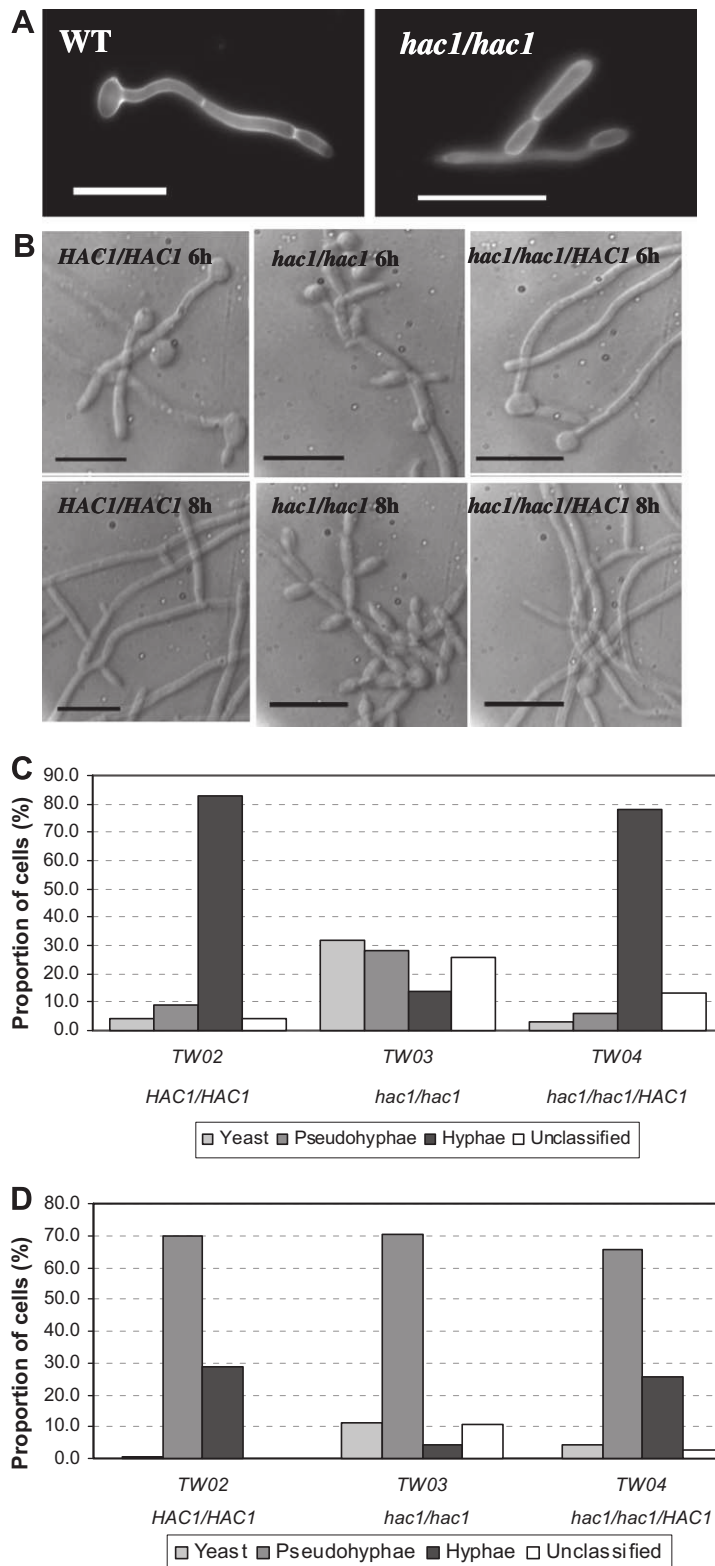


Fig. 8. Effect of inactivating Hac1 upon filamentation in *C. albicans*. (A) WT (BWP17) and *hac1/hac1* (TW03) strains grown in YPD containing 20% (v/v) serum for 5 h, stained with 0.1% Calcofluor white, and visualized by fluorescence microscopy. Scale bar = 20 μ m. (B) WT, *hac1/hac1* and *hac1/hac1/HAC1* strains 6 and 8 h after inoculation in YPD with 20% (v/v) serum at 37 $^{\circ}$ C. Scale bar = 20 μ m. (C) Distribution of the percentage of hyphal-cell forms calculated using Mi in WT, *hac1/hac1* and *hac1/hac1/HAC1* strains in YPD with 20% (v/v) serum at 37 $^{\circ}$ C at 6 h after inoculation (D) Distribution of the percentage of hyphal-cell forms calculated using Mi in WT, *hac1/hac1* and *hac1/hac1/HAC1* strains in Soll's medium (pH 6.5 at 37 $^{\circ}$ C) at 5 h after inoculation. Data represent the average values calculated from each condition for each of duplicate cultures. Cells with Mi from 1.5 to 2.5 were represented as unclassified, because they were not identifiable as yeast, pseudohyphae or hyphae (Brown, 2002). (E) WT, *hac1/hac1* and *hac1/hac1/HAC1* cells in YPD with 20% (v/v) serum at 37 $^{\circ}$ C, 8 h after inoculation. Scale bar = 20 μ m.

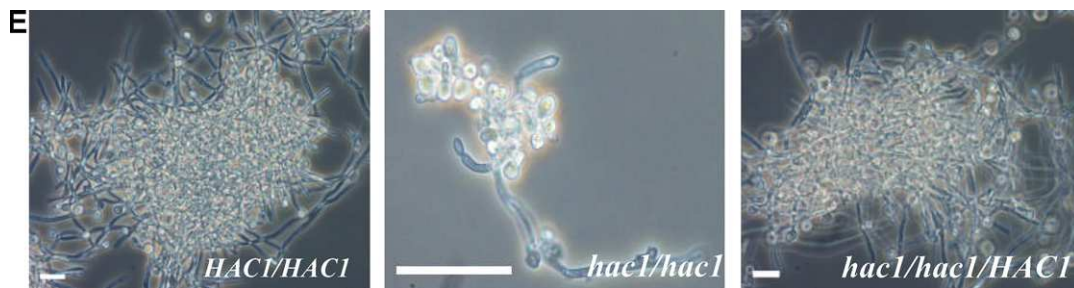


Fig. 8 (continued)

on the regulation of *HAC1* orthologues in other fungi (Cox and Walter, 1996; Saloheimo et al., 2003). We also showed that ER stress gives rise to *C. albicans HAC1* transcripts with truncated 5' UTRs. This phenomenon is similar to that in filamentous fungi and contrasts with *HAC1* regulation in *S. cerevisiae*. Our complementation studies confirmed that *C. albicans Hac1* is a functional homologue of *S. cerevisiae Hac1p*, and showed that *S. cerevisiae* is unable to functionally express unspliced versions of the *C. albicans HAC1* sequence. This reinforces the view that *C. albicans HAC1* transcripts are processed in a manner that is more similar to filamentous fungi than *S. cerevisiae*.

The microarray studies have defined the genome-wide transcriptional response of *C. albicans* to the ER stresses DTT and tunicamycin (Supplementary Data, Part A). This pathogen responds in a similar manner to *S. cerevisiae* (Kimata et al., 2005; Travers et al. 2000), namely by up-regulating genes involved in secretion, vesicle trafficking and protein folding, and down-regulating ribosome biogenesis and protein synthesis. Equivalent functions are regulated by the UPR in yeasts and filamentous fungi (Schröder and Kaufman, 2005a; Travers et al., 2000; Kimata et al., 2005; Sims et al., 2005; Guillemette et al., 2007). *Hac1* makes a significant contribution to the genome-wide transcriptional response to ER stress in *C. albicans*. The proportion of genes that was induced (≥ 1.5 -fold) by both DTT and tunicamycin, and dependent on *Hac1*, was 20.1%. Affected functions include translocation to and from the ER, vesicle transport, membrane lipid metabolism, protein glycosylation and ER-associated protein degradation (ERAD). *IRE1* was not induced by DTT or tunicamycin and neither were any *DER* genes (involved in ERAD). However, there are some interesting differences between *C. albicans* and *S. cerevisiae*. Only 12 genes identified as being *Hac1*-regulated under stress conditions in *C. albicans* were also found to be *Hac1p*-regulated under stress in *S. cerevisiae*. Different criteria were used to identify the UPR genes in *S. cerevisiae* (Travers et al., 2000) so comparisons between the datasets are not straightforward. Although Travers et al. (2000) have provided datasets that catalogue the role of *HAC1* in stress responses in *S. cerevisiae*, there are no equivalent data comparing WT and *hac1* cells in the absence of ER stress. We undertook that analysis in *C. albicans* but, as expected, the results from the array studies did not suggest a dramatic impact on transcription. Under normal growth conditions, the level of *HAC1*ⁱ mRNA (leading to the synthesis of *Hac1p*) is relatively low and the turnover rate of *Hac1p* is high (Kawahara et al., 1997). It is only under stress conditions that the level of *Hac1p* is elevated, and where an impact on transcription of target genes that is detectable by arrays, is expected. Even so, the expression of several secretion-related genes was shown to be *Hac1*-dependent in *C. albicans* and we showed a clear morphological dependence on *Hac1* (below).

Given the significant contribution of *Hac1* to the genome-wide transcriptional response of *C. albicans* to ER stresses, one might expect the resistance of this organism to such stresses to be compromised following *Hac1* inactivation. This was the case, confirming

the central role of *Hac1* in the *C. albicans* UPR. A previous study has inferred that *HAC1* might be an essential gene in *C. albicans* because it was not possible to generate a homozygous mutant using a split-marker transposon mutagenesis strategy (Nobile and Mitchell, 2005). We demonstrate here that homozygous *C. albicans hac1/hac1* null cells are viable, albeit displaying a range of cellular and molecular phenotypes. This is consistent with the situation in other fungi, where *HAC1* orthologues are not essential for viability, but do play central roles in the UPR (Cox and Walter, 1996; Saloheimo et al., 2003).

Candida albicans hac1/hac1 cells displayed sensitivities to Calcofluor white and Congo red, which interfere with chitin and glucan synthesis and cross linking, respectively. Sensitivity to Congo red and Calcofluor white is a phenomenon often observed in glycosylation-deficient and cell wall-defective strains (Bates et al., 2005; Ram and Klis, 2006). These phenotypes are consistent with the concept that *Hac1* inactivation compromises the integrity of the *C. albicans* cell wall even if not all aspects of cell wall biogenesis are compromised. Basal ER trafficking and cell wall functions must be retained in *hac1/hac1* cells because they are viable. We have shown that *Hac1*-regulated functions contribute to the polarized growth of *C. albicans*. *Hac1* is not essential for hyphal development because some hyphae are formed by *C. albicans hac1/hac1* cells under the conditions examined (serum, and variation in pH and temperature). Nevertheless, formation of hyphae is inhibited in *hac1/hac1* cells under these conditions, possibly through inhibition of vesicular trafficking, which is required for polarized growth (Crampin et al., 2005), and the expression of some secretion-related genes was *Hac1*-dependent under non-stress conditions. The transition from yeast to pseudohyphal cells was shown to be *Hac1p*-dependent in *S. cerevisiae* (Schröder et al., 2000) but ours is the first demonstration of the importance of *Hac1* in a fully filamentous organism and one where virulence is related to hyphal development. *hac1* mutant cells display this hyphal defect in the absence of ER stress under conditions where *HAC1* mRNA splicing was not detected. It is formally possible that splicing is not required for *HAC1* mRNA to mediate its effects upon polarized growth. However, we favour the idea that basal levels of *HAC1* mRNA splicing are sufficient to promote polarized growth in the absence of ER stress.

The impact of *HAC1* upon hyphal development in *C. albicans* is significant for several reasons. Firstly, hyphal development contributes to the virulence of this pathogen (Lo et al., 1997; Gow et al., 2002, 2003; Saville et al., 2003; Zheng et al., 2004; Sundström, 2006). Hyphal development in *C. albicans* is regulated through several signalling pathways including the Ras-cAMP and MAPK pathways (Lo et al., 1997; Ernst, 2000; Brown, 2002). Polarized growth can also be stimulated by amino acid starvation via the bZIP transcription factor, *Gcn4* (Tripathi et al., 2002). *Hac1p* and *Gcn4p* act in concert to control the expression of some UPR genes in *S. cerevisiae* (Patil et al., 2004). Therefore *C. albicans Hac1* might mediate its effects upon polarized growth independently, or in concert with

other transcription factors such as Gcn4. Secondly, the yeast–hyphal morphogenetic transition is characterized by alterations in the composition of the *C. albicans* cell wall, which include changes in chitin content and the differential regulation of *ALS* (agglutinin-like sequence) adhesin genes (Munro et al., 1998; Hoyer, 2001; Zhao et al., 2004). The synthesis, delivery and regulation of cell surface molecules are known to contribute to the virulence of *C. albicans* (Staab et al., 1996, 1999; Bates et al., 2005, 2006). The impact of Hac1 in *C. albicans* is evident from its effect on morphology. The transcriptional dependencies on Hac1 are most evident under ER stress conditions and it is possible that ER stress occurs during invasive growth of *C. albicans*.

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Appendix A. Supplementary data

Supplementary data associated with this paper is available to download online at www.ebi.ac.uk/arrayexpress (E-MEXP-1307, Wimalasena 2007). Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.fgb.2008.06.001](https://doi.org/10.1016/j.fgb.2008.06.001).

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