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Cell surface changes in the *Candida albicans* mitochondrial mutant *goa1Δ* are associated with reduced recognition by innate immune cells

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Summary

We have previously characterized several fungal-specific proteins from the human pathogen *Candida albicans* that either encode subunits of mitochondria Complex I (CI) of the electron transport chain (ETC) or regulate CI activity (Goa1p). Herein, the role of energy production and cell wall gene expression is investigated in the mitochondria mutant *goa1*Δ. We show that down regulation of cell wall-encoding genes in the *goa1*Δ results in sensitivity to cell wall inhibitors such as congo red and calcofluor white, reduced phagocytosis by a macrophage cell line, reduced recognition by macrophage receptors, and decreased expression of cytokines such as IL-6, IL-10, and IFN-γ. In spite of the reduced recognition by macrophages, the *goa1*Δ is still killed to the same extent as control strains. We also demonstrate that expression of the epithelial cell receptors E-cadherin and EGFR is also reduced in the presence of *goa1*Δ. Together, our data demonstrate the importance of mitochondria in the expression of cell wall biomolecules and the interaction of *C. albicans* with innate immune and epithelial cells. Our underlying premise is that mitochondrial proteins such as Goa1p and other fungal-specific mitochondrial proteins regulate critical functions in cell growth and in virulence. As such, they remain as valid drug targets for antifungal drug discovery.

Keywords

cell wall; mitochondria; immune recognition; macrophages; *Candida albicans*

Introduction

Candida albicans is a member of the commensal microbiota of mucosal surfaces. In HIV/AIDS patients, infections are usually limited to the oral, pharyngeal, and esophageal mucosa, while blood-borne invasive disease occurs in patients with risk factors such as surgery, neutropenia, and indwelling urinary track and central venous catheters (Rüping *et*

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al., 2008; Leventakos *et al*., 2010; Kriengkauykiat *et al*., 2011; Williams *et al*., 2011). Neutropenia occurs as a result of immune suppression previous to allogeneic bone marrow transplants or cancer chemotherapy. The reversible transition of the organism from a unicellular growth form to one that is filamentous has long been recognized as contributing to the invasiveness of the organism (Calderone *et al*., 2001). Also, cell surface adhesins of *C. albicans* are critical to the colonization of tissues. Thus, the ability to transform from a unicellular yeast into a multicellular, hyphal state, to adhere to, and invade a variety of tissues and immune cells, as well as utilizing strategies to evade immune protection are considered as the virulence factors for developing candidiasis (Brand *et al*., 2012).

The host protective response to commensal organisms such as *Candida albicans* relies on innate and cell-mediated immunity (Brown *et al*., 2012). Polymorphonuclear neutrophils (PMNs) and macrophages are both important for innate immune responses, and T-helper (Th) cell-mediated responses are also critical to protection against fungal infections (Romani *et al*., 2000; Lionakis *et al*., 2010). For fungal pathogens, the innate immune system recognizes conserved cell wall biomolecules such as mannan, β-glucan, and chitin (Porcaro *et al*., 2003). The recognition of fungal cells via receptors on these two types of immune cells is followed by the release of proinflammatory cytokines, such as interleukin (IL)-1, IL-6, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, and the activation of a respiratory burst following phagocytosis (Kim *et al*., 2005). Among the receptors for fungal cell wall oligosaccharides, dectin-1, toll-like receptors TLR-2 and TLR-4, seem to be most often studied. However, experiments on receptor-fungal cell wall ligands indicate that receptor interactions are required for recognition of a single ligand (Netea *et al*., 2006; Gow *et al*., 2007).

Endocytosis of *C. albicans* by epithelial and endothelial cells was suggested as a mechanism to provide access to the dermal basement membranes and blood vessels, which eventually results in systemic dissemination (Filler *et al*., 1995). To bind to a variety of host cells, *C. albicans* produces a number of cell surface adhesins. Among the most well-known of these is the ALS (agglutinin-like sequence) gene family, a group of glycoproteins that was first found to participate in a mating-associated adhesion event in *S. cerevisiae* (Draninis *et al*., 2007). In *C. albicans*, the roles of some of the ALS adhesins in oropharyngeal candidiasis and in the onset of endocytosis have been well established (Green *et al*., 2004; Cheng *et al*., 2005; Hoyer *et al*., 2007). For example, the Als3p of *C. albicans* mediates endocytosis by interacting with cadherins of epithelial and endothelial cells (Phan *et al*., 2007).

Oppositely, *C. albicans* has evolved ways to escape the host immune system and progress to a pathogenic status following initial host cell interactions. One such mechanism is the hyphal transition, during which the organism converts to a filamentous growth post-phagocytosis in macrophages to exit these cells, and invade tissues (Sudbery et al., 2004).

The morphological switch from yeast to filamentous forms can be introduced by a variety of environmental conditions that require signal transduction pathways. In addition, phagocytosis and outgrowth by hyphae from macrophages has been studied in the context of metabolic changes occurring during the intracellular phase of *C. albicans* (Lorenz et al., 2004). Coordination of intracellular events following its phagocytosis with nutrient availability and oxygen radical levels in the macrophage occurs. Once internalized by macrophages, during early stage events, the organism begins a low nutrient growth program, characterized by gluconeogenesis, β-oxidation of fatty acids, and the conservation of carbon via the glyoxylate cycle. These events require crosstalking of peroxisomes and mitochondria in *C. albicans*. As hyphal transition follows and the organism initiates outgrowth from the macrophage, its metabolism changes to a glycolytic metabolism (Lorenz et al., 2004).

Therefore, and not surprisingly, the switch to a hyphal morphology in *C. albicans* depends upon regulated metabolic events that include carbon metabolism.

In this study, we focus upon mitochondrial events that influence interactions with macrophages and epithelial cells. Using a well-characterized mitochondrial mutant lacking the gene *GOA1*, we have previously shown its role in maintaining membrane potential, ATP synthesis, reduction of toxic levels of cell ROS, and virulence. We also know that during oxidant stress, Goa1p translocates to the mitochondria and regulates Complex I (CI) of the electron transport chain (ETC) (Bambach *et al.*, 2009; Li, *et al.*, 2011; Chen *et al.*, 2012). Herein, we report the cell surface changes in *goa1*Δ and the consequences of those changes on the recognition by macrophage and epithelial cell receptors.

Results

Mitochondria of *C. albicans* are required for cell wall maintenance and expression of adherence genes

The cell wall of *Candida* species consists of an inner layer adjacent to the plasma membrane composed mostly β-glucans and chitin, which provide a rigid cell structure and an outer fibrillar layer that is mainly composed of mannan and glycoproteins such as those of the ALS adherence family. Covalent attachment to either β-1,3 or β-1,6 glucan anchors glycoproteins to the wall matrix. We previously reported a significant down regulation of 86 genes (5.38% of total) associated with cell wall functions in the *goa1*Δ compared to its parental strain (Sun *et al.*, 2013). Of these, 40 genes are associated with cell wall biogenesis and integrity, 26 genes encode adhesin proteins that included 7 of the ALS gene family, and 14 genes that are required for the yeast-hyphal switch (**summary, Table 2**). Also available for analysis was the *ndh51*Δ and reintegrant strain. *NDH51* encodes a subunit of the CI ETC. The total number of down regulated genes associated with cell wall functions in this mutant is approximately the same as with the *goa1*Δ. Of these, both mutants display similar wall-associated gene changes, indicating a crucial role for mitochondria in these processes (data not shown).

Based upon these data, we compared the sensitivities of strains to Congo red (CR) and calcofluor white (CFW), cell flocculation, and cell wall hydrophobicity. As shown in **Table 2**, a decrease in genes encoding a mannosyltransferase (*MNN4. MNT4*) and glucan synthases (*FKS1, ENG1, PHR1, PHR3*) correlates with the sensitivity of *goa1*Δ to CR and CFW (**Fig. 1A)** in contrast to the wild type (WT) and reconstituted strain (GOA32). Similar growth inhibition by CR and CFW was noted with *ndh51*Δ compared to its integrant strain (Ndh51R) (**Fig. 1B**). The *goa1*Δ also flocculated much more extensively in RPMI after 6 h **(Fig1.C)** and had increased hydrophobicity (**Fig. 1D**). The sensitivity to cell wall inhibitors, increased hydrophobicity ($p<0.01$), and increased flocculation demonstrate that major cell wall changes have occurred in the mutants.

Mitochondrial mutant *goa1Δ* is phagocytosed poorly by mouse phagocytes

From previous published data, Goa1p is required for mitochondrial complex I (CI) activity, cristae integrity, and oxidative phosphorylation (Li, *et al.*, 2011; Chen *et al.*, 2012). The *goa1*Δ was also avirulent in a blood-borne, invasive candidiasis murine model (Bambach *et al.*, 2009). *In vitro* studies indicated an increased killing of the mutant by polymorphonuclear neutrophils even though it was phagocytosed less than control strains (Bambach *et al.*, 2009). To continue our studies of the *goa1*Δ and interactions with host cells, we used a mouse macrophage cell line to measure phagocytosis **(Fig. 2A and 2B)**. In agreement with reduced gene expression of cell wall components, phagocytosis by the macrophage cell line RAW264.7 was reduced by approximately 50% after 30 min for the

goa1Δ mutant compared with WT and GOA32 strains, $p < 0.001$ (**Fig. 2B)**. We also measured macrophage killing of *goa1Δ*, WT and the *GOA1*-reconstituted strain (GOA32) after 24 h of incubation. Unlike our previous studies with PMN, we found no significant differences in killing of all strains by macrophages **(Fig. 2C)**. These results indicate that *goa1*Δ is defective in its ability to induce phagocytosis by phagocytes, although its killing by phagocytes was not impaired. Our interpretation of these data is that extracellular killing by macrophages of *goa1*Δ accounts for the similar levels of killing in all strains. The anti-*Candida* killing by PMNs but not equally by macrophages is well-known (Miramón, *et al.*, 2012).

Cytokine production by macrophages is reduced in the presence of *goa1Δ*

In addition to the innate immune response against *C. albicans* infections, cellular immune responses that require the activation of $CD4^+$ (Th-1) cells are also critical. The outer mannan polysaccharides, glycoproteins of the *C. albicans* cell wall, and inner layer of β-glucan are recognized by innate immune cells via their cell surface receptors. Since many genes encoding these polymers in *goa1*Δ are down regulated (**Table 2**), we measured TLR-2, TLR-4 and dectin-1 expression after incubation of all strains with the same macrophage cell line. Although synergized responses of cytokine release have been reported (Ferwerda, *et al.*, 2008), the Toll-like receptors TLR-2 and TLR-4 bind mannan while dectin-1 recognizes β-glucan mostly.

In addition to the three macrophage receptors that recognize fungal biomolecules, we also measured downstream pro-inflammatory cytokines such as TNF-α, IL-10, IL-6, IFN-γ, and MyD88 in the presence of *goa1*Δ and control strains after 1 and 2h of incubation (**Fig. 3A,B)**. Expression of TLR-2 and TLR4, was significantly reduced after 1 h in the presence of *goa1Δ* compared to WT and GOA32 ($p < 0.001$). No differences were observed in dectin-1 expression among strains after 1 h of incubation. After 2 h of incubation, the expression of TLR-2 and TLR-4 remained lower in *goa1*Δ, and while expression of dectin-1 was low for all strains, less was observed in *goa1*Δ (**Fig. 3A,B**).

Cytokines IL-10 and IL-6 were significantly reduced in *goa1Δ* compared to control strains, but TNF-α remained at comparable levels in all strains ($p = 0.10$ at 1h and $p = 0.02$ at 2h) **(Fig.3A,B)**. A significant reduction of IL-6 was also confirmed by ELASA ($p < 0.005$) **(Fig. 3C)**. IFN-γ, released by the activation of Th-1 cells and which also plays a major role in activating phagocytic cells against *Candida*, was reduced in *goa1*Δ as was the adapter protein MyD88 (Marr *et al.*, 2003), which mediates a signaling pathway that results in translocation of NF-κB and subsequent cytokine genes **(Fig 3A,B)**.

The most dramatic differences in cytokine profiles among strains were that of IL-6 and IL-10. Both cytokines were decreased by 20-fold at 1 h in *goa1*Δ. IL-6 is one of the cytokines necessary for the development of Th17, a T-cell subset essential for immunity to *C. albicans* (Ghosh, *et al.*, 2010). IL-6 and IL-10 are both anti-inflammatory cytokines that are secreted by T-cells and macrophages whose activation, in general, inhibits macrophage pro-inflammatory cytokine production, such as IFN-γ, TNF-α, and also blocks NF-κB activity. Correspondingly, we observed less expression but still differences in IFN-γ, TNF-α and MyD88 expression profiles between mutant and control strains.

Decreased recognition and endocytosis of *goa1Δ* by human epithelial cells

Invasion of epithelial cells follows colonization by *C. albicans* in candidiasis patients. *C. albicans* binds to the epithelial receptors E-cadherin (calcium-dependent adhesion) and EGFR (epidermal growth factor receptor) via its adherence proteins Als3 and Ssa1p (Zhu *et al.*, 2012) and induces endocytosis. To determine if reduced expression of adherence

glycoproteins in *goa1*Δ (**Table 2**) affected epithelial endocytosis, we examined the distribution of epithelial receptor E-cadherin and EGFR by confocal microscopy after infection of the FaDu oral epithelial cell line with *goa1*Δ and control strains. As shown at **Fig. 4**, both receptors were more highly expressed in WT or GOA32 than when infected with *goa1Δ*. Fluorescence was more intense on yeast buds and germinating hyphae of control strains, whereas fluorescence was less on mother cells, or partially covered the germ tubes of the *goa1*Δ mutant. This observation was also confirmed by a significant reduction in the expression of receptors by the epithelial cell line to *goa1Δ*, as determined by qPCR after infection for 1 h (**Fig. 5A)**. Accordingly, less endocytosis of *goa1* was observed compared to control strains (**Fig. 5B**). As Als3p and Ssa1p are important adhesin glycoproteins that promote binding to epithelial cells, we also measured the expression of both in *goa1*Δ compared to control strains (**Fig. 5C, D**). Both genes were down regulated in the *goa1*Δ in YPD (Table 2) or 10% BSA.

Discussion

Previously, we have shown that *GOA1* from *C. albicans* is required for normal mitochondrial functions. The lack of *GOA1* caused a major reduction in respiration associated with a decrease in both CI activity of the ETC and oxidative phosphorylation and an increase in cell ROS. As a consequence, the mutant had a shortened chronological aging and cell apoptosis, changes that were probably of consequence to a reduction in virulence and colonization of the kidney in a murine model of blood-borne candidiasis (Bambach *et al*, 2009). *In vitro* experiments demonstrated a greater killing by human neutrophils of *goa1Δ*. We have also noted a down regulation of mitochondria and peroxisomal genes that likely indicate an inability of these two organelles to cross-talk in shared pathways such as gluconeogenesis, fatty acid β-oxidation, and the glyoxylate bypass pathway (Li, *et al.*, 2011; Chen *et al.*, 2012; Sun *et al.*, 2013). The ability of *Candida albicans* to adapt to carbohydrate starvation, nitrosative stress, and oxidative stress during phagocytosis is essential for its virulence (Miramón, *et al.*, 2012).

Our current data indicate a failure by macrophages and epithelial cells to fully recognize the mutant. Our explanation is that decreased expression of genes associated with cell wall biosynthesis and integrity, including adhesins, are contributory to reduced recognition of the mutant. Cell surface changes were initially shown as an increased sensitivity of the mutant to cell wall perturbing compounds such as Congo red and calcoflour white. Heightened sensitivity of the mutant compared to control strains was associated with reduced expression of 72 genes encoding proteins associated with cell wall biogenesis, organization, and adherence proteins such as the ALS family of glycoproteins. Cell surface changes should have an effect on host-fungus interactions, now clearly observed in our current report. Our data indicate that mitochondria/peroxisomes play an important role in the biosynthesis and organization of the fungal cell wall that also has implications in host recognition (Chen, *et al.*, 2012; Sun, *et al.*, 2013).

The fungal cell wall is a highly dynamic structure that changes in regard to environmental conditions such as nutrient limitation. Fungal cell wall ligands for dectin-1, TLR-2 and TLR-4 are β-glucan, phospholipomannan, and *O*-linked mannans, respectively (Lionakis, *et al.*, 2013). Dectin-1 expression in macrophages was lower for all strains at 1h due perhaps to its inner cell wall localization, while TLR-2 expression was significantly reduced in macrophages at 1-2 h post-infection. Among the three receptors, down regulation of TLR-2 leads to a significant reduction in IL-6 and IL-10 production in *goa1*Δ since TLR-2 is responsible for pro-inflammatory and anti-inflammatory cytokine expression. However, TNFα, a downstream cytokine of TLR-2, was maintained at sufficient levels in *goa1*Δ by 1h. Cell wall mannosylation is a key determinant in phagocytosis, since glycosylation

mutants have delayed endocytosis when encountering macrophages (Lewis, *et al.,* 2012). Therefore, with regard to the deficiencies in mitochondria in *goa1*Δ, cell surface phospholipomannan for TLR-2 may be more important to mediate host-organism interaction and recognition by macrophages. We cannot determine precisely as yet how the loss of *GOA1* affects the proportion of cell wall polysaccharides.

In addition to the defects in recognition of the mutant, we have previously shown that the *goa1*Δ as well as the *ndh51*Δ are hypersusceptible to azoles, including fluconazole, but have wt sensitivity to micafungin and amphotericin B (Sun *et al*, 2013). Ndh51p is a subunit protein of the ETC complex 1 (CI). Thus, both a CI regulatory protein and a CI subunit protein have similar antifungal drug susceptibilities. The influence of another mitochondrial mutant on its antifungal susceptibility has been reported. The mitochondrial outer membrane protein Sam37p (Sorting and Assembly Machinery) is required for cell wall integrity and is essential for caspofungin tolerance (Dagley et al, 2011). The *sam37*Δ though, had no cell wall changes in the relative amounts of carbohydrate compared to control strains (Qu *et al*, 2012). However, *CCR4-Pop2*, an mRNA deadenylase and a regulator of mRNA stability and translation is required for cell integrity, mitochondrial functions, and phospholipid balance (Dagley *et al*., 2011). The corresponding mutant had reduced levels of cell wall β-glucans and was sensitive to caspofungin. Therefore, a relationship between trafficking of phospholipids and cell wall integrity has been suggested. In this regard, Singh *et al*, 2012, report that changes in specific phospholipid classes of the mitochondrial and plasma membranes of *C. albicans* were associated with a 200-fold increase in fluconazole resistance. An explanation for cell surface changes and antifungal susceptibility is the reduced membrane phospholipid content in certain mutants. The underlying changes in the *goa1*Δ cell wall may be different from that described in the *sam37*Δ and *ccr4-pop2Δ* mutants based upon their hypersusceptibility profiles mentioned above implying gene-specific affects on tolerance.

Nevertheless, the sensitivity to fluconazole may also suggest that a membrane alteration occurred in *goa1Δ* and the *ndh51*Δ. One of intermediate products of phospholipid homeostasis is phosphatidylinositol (PI) that facilitates ER, mitochondrial and plasma membrane homeostasis. PI is also a constituent of cell surface GPI-anchored glycoproteins such as the ALS family of GPI-anchored adhesins (Richard, *et al*., 2002). However, as mentioned above, the type of cell wall change may depend upon the specific mitochondrial defect.

The cell wall changes in *goa1*Δ caused a decrease in its recognition by cells such as macrophages. However, the mutant is killed to the same extent as control strains. How does this occur? The increased killing of the mutant can be at least partially explained by its changes in carbon metabolism previously published (Chen, *et al*., 2012; Sun, *et al*., 2013). Mitochondria usually provide cells with energy through oxidative phosphorylation when grown in nutrient-rich media. However, because of near starvation conditions in macrophages, especially in regard to glucose, during phagocytosis, growth slows, alternate carbon sources are utilized, and oxidative stress responses are induced (Miramón, *et al*., 2012). Subsequently, yeast-hyphal transition occurs allowing escape of *C. albicans* from macrophages. Accompanying or prior to morphogenesis is an activation of glycolysis/ mitochondrial respiration and a down regulation of stress responses. The metabolic pathways for alternate carbon utilization during reduced glucose include those that are compromised in the mutant as outlined above. Thus, the necessary metabolic switch that allows wild type cells to adapt and escape macrophages is very likely absent in the mutant (Sun, *et al*., 2013).

It is quite likely that other deficiencies in the mutant result in killing by macrophages. We speculate that increased ROS in the mutant caused by dysfunctional mitochondria complex I (CI) (Li *et al.*, 2011) may also contribute to its death. Thus, heightened mutant cell ROS as well as that produced by macrophages (both internal and external) may be another reason for cell death in *goa1*Δ. Consequently, the increase in killing of the mutant by macrophages (this study) and neutrophils (Bambach *et al.*, 2009) may be partially explained by a down regulation of anti-oxidative enzymes and an inability to regulate a response to ROS (Chen, *et al.*, 2012; Sun, *et al.*, 2013).

Quite importantly, Goa1p or its transcriptional regulators could constitute targets that may be exploited in antifungal drug discovery. Preliminary data, in fact, indicate that a transcriptional regulator of *GOA1* expression is fungal-specific. In fact, a new anti-*C. albicans* compound that targets mitochondria has been described (Nishikawa, *et al.*, 2010).

Experimental procedures

Candida Strains and growth conditions

C. albicans strains *goa1Δ* mutant (GOA31) and the gene-reconstituted strain (GOA32) that were previously described were used for all experiments in this study. In addition, wild type SC5314 (WT) was used as a control (Bambach *et al.*, 2009). The *ndh51*Δ and reconstituted strains were used in inhibitor studies. For most experiments, cells were initially grown at 30°C in YPD overnight.

Cell wall phenotypes

Drop plate and cell clumping assays were used to identify cell wall defects in *goa1*Δ. For drop plate assays, the overnight cultures of all strains were centrifuged, washed twice in saline, adjusted to $1\times10^6 - 1\times10^1$ in 5μl, and spotted onto YPD agar medium with or without cell wall inhibitors. The cell wall inhibitor Congo red (CR) or calcofluor white (CFW) each was added to YPD at 25μg/ml. The sensitivity of strains to the cell wall inhibitors was determined after 48h on plates at 30°C. RPMI medium (Invine Scientific) was used to measure cell clumping (Li, *et al.*, 2009). Overnight cultures were collected, washed, and transferred into RPMI media at an OD_{600} of 0.5, then incubated at 37°C for 6h with gentle shaking. Suspensions were vortexed for a few seconds prior to taking photographs.

Cell surface hydrophobicity (CSH)

Cyclohexane and xylene were applied to measure CSH for each strain (Gelis, *et al.*, 2009). Overnight culture were grown to their exponential phase, washed three times with PBS, and adjusted at OD_{600} to 1.0 in 3 ml. The cell suspensions were mixed well with 150 μl of cyclohexane or xylene in acid-washed glass tubes. Cells were incubated at 30°C for 10 min, vortexed once for 1 min, and then transferred to room temperature for 20 min (A0). The absorbance of aqueous phase cells at OD_{600} were measured compared to cell suspensions prior to mixing with each hydrocarbon (A1). The cell surface hydrophobicity was determined by the percentage of (A1/AO)/100. Triplicate readings of samples were averaged for each strain.

Macrophage assays

The mouse macrophage cell line RAW264.7 (ATCC) was used for all assays described below. Cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100U/ml penicillin and 100μg/ml streptomycin at 37°C with 5% CO_2 in air atmosphere.

(i) Macrophage phagocytosis—RAW264.7 confluent cells were collected, washed, and seeded in 96-well plates at 5×10^5 in 100μl per well for 2 h. Following their attachment, cells from overnight cultures of *Candida* strains (1×10^8) were labeled with fluorescein isothiocyanate (FITC, Sigma, 1.25mM in 0.1mM sodium bicarbonate buffer with 0.5% DMSO, pH9.0) at 4°C overnight (Klippel, *et al.*, 2010). After three PBS washes to remove unbound dye, labeled yeast cells suspended in DMEM + 10% FBS in 100μl were added at a ratio of macrophage:yeast=1:2, and plates were incubated for 1 h at 37°C in a 5% CO_2 atmosphere. At each time point, 100μl of cell suspension was removed and 100 μl trypan blue (250μg/ml in PBS) was added to quench the fluorescence of yeasts which were bound but not internalized at room temperature for 1 min. The number of internalized and fluorescent yeasts was then estimated (Ex/Em=495/525) by a 96-plate reader (CytoFluor Series 4000, PerSeptive Biosystems), and verified by fluorescence microscopy as well.

(ii) Macrophage killing assay—As stated above, macrophage RAW264.7 cells (1 day old) were prepared and seeded in a 96-well plate at 5×10^4 in 150μl of DMEM overnight at 37°C as described above. The overnight cultures of strains were diluted into DMEM at 2×10^6/ml, and a 50μl aliquot of each strain was added into the first column wells, mixed with macrophages, and then serially diluted 1:4 a total of six times. Strains incubated without macrophages were diluted similarly. Plates were incubated for 24h at 37°C in a 5% CO_2 atmosphere. Then, colonies were counted by microscopy in each well. The survival rate of each strain was calculated as the numbers of colonies with macrophages divided by the number of colonies in the absence of macrophages x100. Each experiment was done in duplicate, and *p* values were determined using the unpaired Student's t test.

(iii) Cytokine expression in macrophages infected with *Candida* strains—The expression of cytokines was measured by real-time PCR. RAW264.7 cells were prepared as previously described (Ghosh, *et al.*, 2010). Briefly, 1×10^6 cells/well were seeded in 6-well plates in the appropriate medium and grown overnight at 37°C in a 5% CO_2 atmosphere. The overnight cultures of all *Candida* strains were washed with PBS, suspended in DMEM, and then added to each macrophage culture at ratio of *C. albicans*: macrophages =4:1. Mixed cultures or controls were then incubated for 1 or 2 h. For all macrophage cytokine assays, cell pellets were washed three times with PBS. RNA extractions were performed by adding 1ml of Trizol (Invitrogen). The quality and concentration of RNAs were measured with a nanospectrophotometer, and approximately 0.8 μg of RNA was used to prepare cDNA. Quantitative real-time PCR (qPCR) was carried out in 20-μl reaction volumes that contained 1× iQ SyBR green Supermix (Bio-Rad), including a 0.2 μM concentration of each primer (**Table 2**), and 8 μl of a 1:8 dilution of each cDNA from each strain. The experiment was performed in triplicate using Bio-Rad iQ5, and the transcription level of each gene was normalized to GAPDH levels. Data are presented as the means ± standard deviations (SD). The $2^{-\Delta\Delta CT}$ (where CT is the threshold cycle) method of analysis was used to determine the fold change in gene transcription (Sun, *et al.*, 2013).

Cytokine production of IL-6 was quantified by ELISA to confirm the expression profile after infection with strains (Ferwerda, *et al.*, 2008). In a 96-well plate, adherent RAW264.7 macrophages (5×10^4 in 100 μl) were infected with 100 μl of yeast cells (1×10^4) in DMEM supplemented with 10% FBS, and co-incubated at 37°C in a 5% CO_2 in air atmosphere for 3 h. As a negative control, macrophages were cultivated without yeasts. After centrifugation of the plates at 4°C for 5 min at 2,250 × g, the supernatants were collected, and stored at -80 °C until assays were performed. IL-6 levels were determined with an ELISA kit (ELISA Ready-SET-Gol, R&D Systems quantikine mouse IL-6 Kit) according to the protocol of the manufacturer. For analysis, background levels from non-infected macrophage cells were measured to obtain final determinations of IL-6 levels of co-cultivated cultures.

Epithelial cell assays

The FaDu epithelial cell line was chosen to measure differences among all *Candida* strains for the expression of epithelial receptors. FaDu cells (purchased from ATCC) were maintained at 37°C with 5% CO_2 in MEM Earl's salts (Irvine Scientific) containing 10% FBS, 1 mM pyruvic acid, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 IU/ml penicillin, and 100 IU/ml streptomycin. Cells were subjected to no more than 15 passages for our study (Phan *et al.*, 2007; Zhu *et al.*, 2012).

(i) Expression of epithelial cell receptors—The expression of epithelial cell EGFR and E-Cadherin around all strains of *C. albicans* hyphae was visualized using direct immunofluorescence. FaDu cells were prepared on fibronectin-coated coverslips in a 24-well tissue culture plate, and incubated with 2.5×10^5 *C. albicans* yeast cells for 90 min. The length of germ tubes and extent of hyphae formation among different strains was recorded. Cultures were fixed and permeabilized with 3% paraformaldehyde containing 0.5% triton X-100 in PBS, rinsed once with 1% BSA in PBS, and blocked with 5% goat serum in PBS. After extensive rinsing, the cells were incubated with either the anti-Ecadherin rabbit monoclonal antibody conjugated with Alexa 488 or an anti-EGFR rabbit monoclonal antibody conjugated with Alexa 555 (Cell Signaling Technology, catalog #3199 and #5108). Imaging was by confocal scanning laser microscopy (Zeiss LSM 510 META). Images of the organisms and host cells were acquired; and three to five optical sections were stacked along the z-axis to produce the final images (Zhu *et al.*, 2012).

(ii) Endocytosis and binding of *C. albicans* by epithelial cells—The number of organisms that were endocytosed or associated with the host cells was quantified. After fixation, epithelial cells were stained with fluorescent conjugated EGFR and E-cadherin. Mixed images of organism and host cells were visualized in 10 high-power fields for each *Candida* strain with confocal scanning laser microscopy. At least 200 organisms were examined on each coverslip, and the results were expressed as the number of endocytosed or cell-associated organisms per high powered field.

(iii) *ALS3* and *SSA1* expression in *C. albicans*—The expression of *ALS3* and *SSA1* were measured by real-time PCR. All strains of *C. albicans* were prepared in YPD or in MEM Earl's salts (Irvine Scientific) containing 10% FBS for 4 hours. RNAs were extracted from each strain following the standard real-time PCR procedure as described above. The corresponding primer sets for each gene were listed in **Table 1**.

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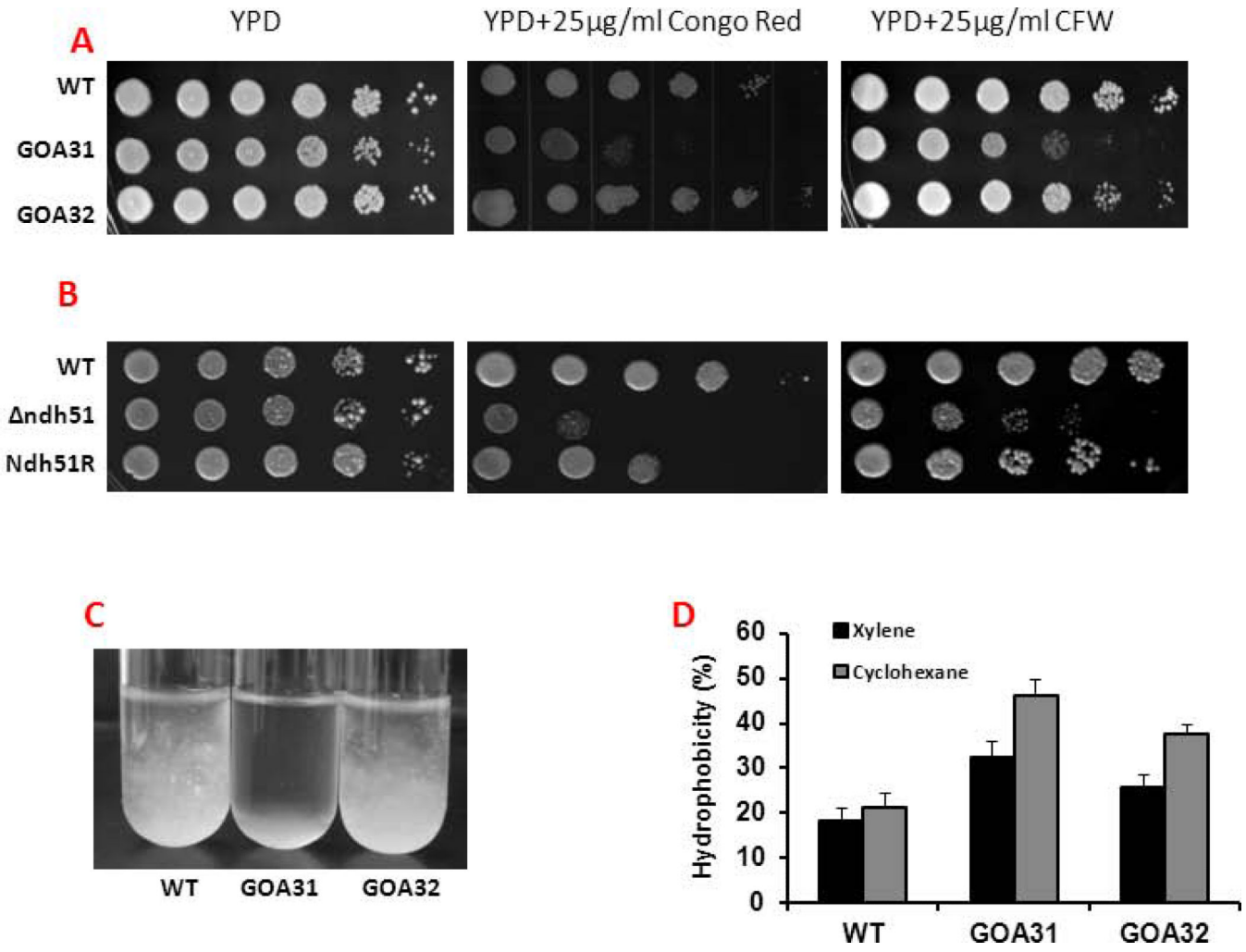

Figure 1.

Deletion of *GOA1* and *NDH51* of *C. albicans* causes changes in its cell wall. **(A)** A cell suspension of WT (SC5314), *goa1Δ (*GOA31) and its gene-reconstituted strain (GOA32) were serially diluted and applied to YPD agar, or YPD agar supplemented with 25 μg/ml of calcoflour white (CFW), or 25 μg/ml of Congo red (CR). Images were taken 48 h after plating cells. The mutant strain was more sensitive than WT and GOA32 to each cell wall inhibitor. **(B)** Similarly, the *ndh51*Δ was also more sensitive to both CR and CFW than control strains **(C)** Flocculation of *goa1*Δ and control strains is compared in RPMI medium. The null mutant flocculated more than control strains. **(D)** Differences in cell surface hydrophobicity with cyclohexane or xylene among strains are represented.

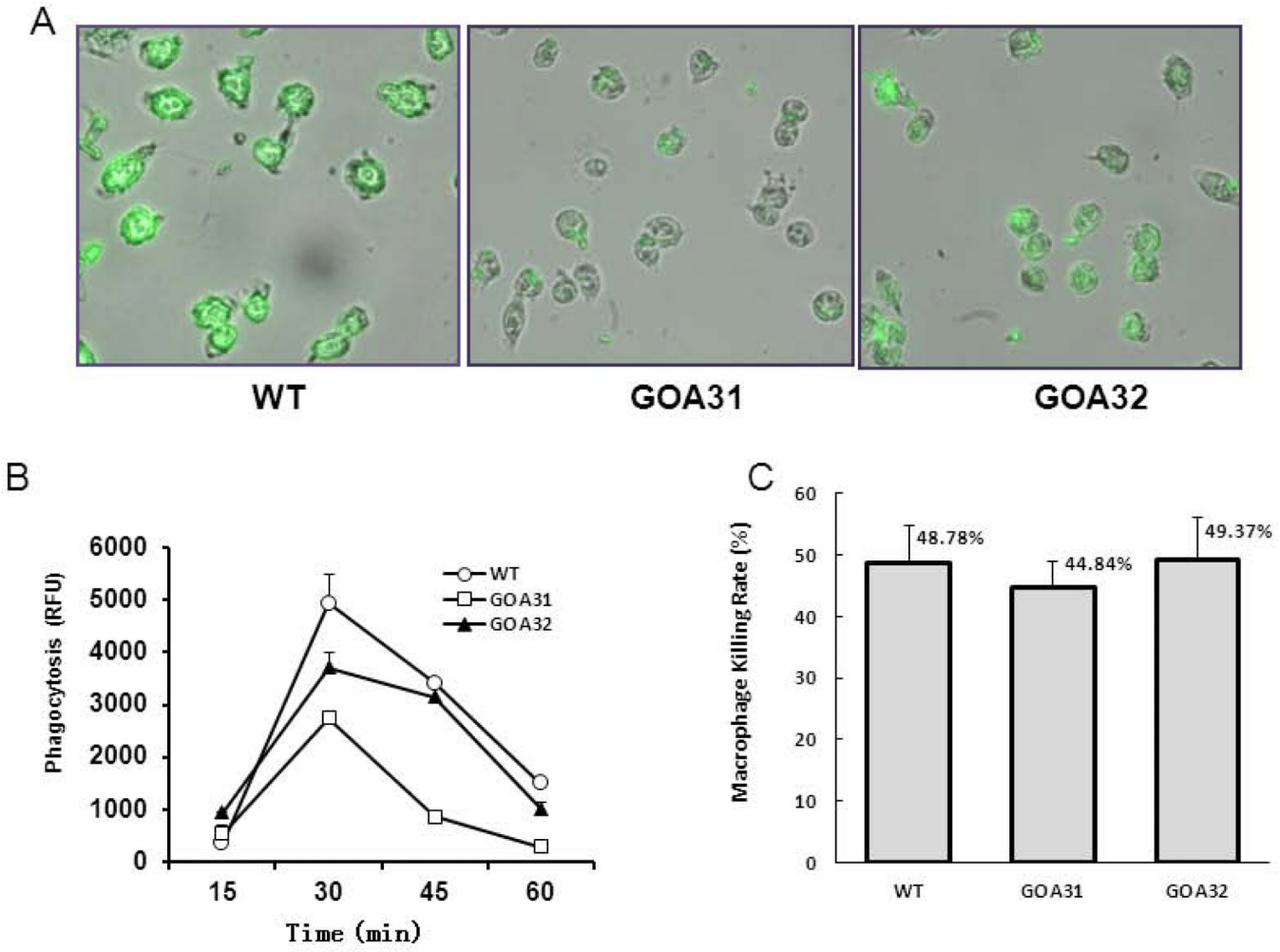

Figure 2.

Phagocytosis of all strains is shown following incubation with macrophages (RAW264.7) for the times indicated. All strains were pre-labeled with FITC. Fluorescence by un-labeled yeast cells was quenched with trypan blue prior to measuring the fluorescence of ingested yeasts. **(A)** Images are displayed from cells following an incubation of 30 min; **(B)** The fluorescence of internalized yeast was followed for 1 h at 15 min intervals by spectrofluorimetrics (Ex/Em=495/525). A reduction in the phagocytosis of *goa1Δ* (GOA31) compared to WT and GOA32 was noted at 30 min ($P < 0.01$). **(C)** Viability of all strains is shown after an overnight incubation with macrophages (RAW264.7). Viable colonies of each strain were counted under microscopy. Data are averages of two separate experiments. A Student's unpaired *t* test was used to determine *P* values, $P > 0.05$.

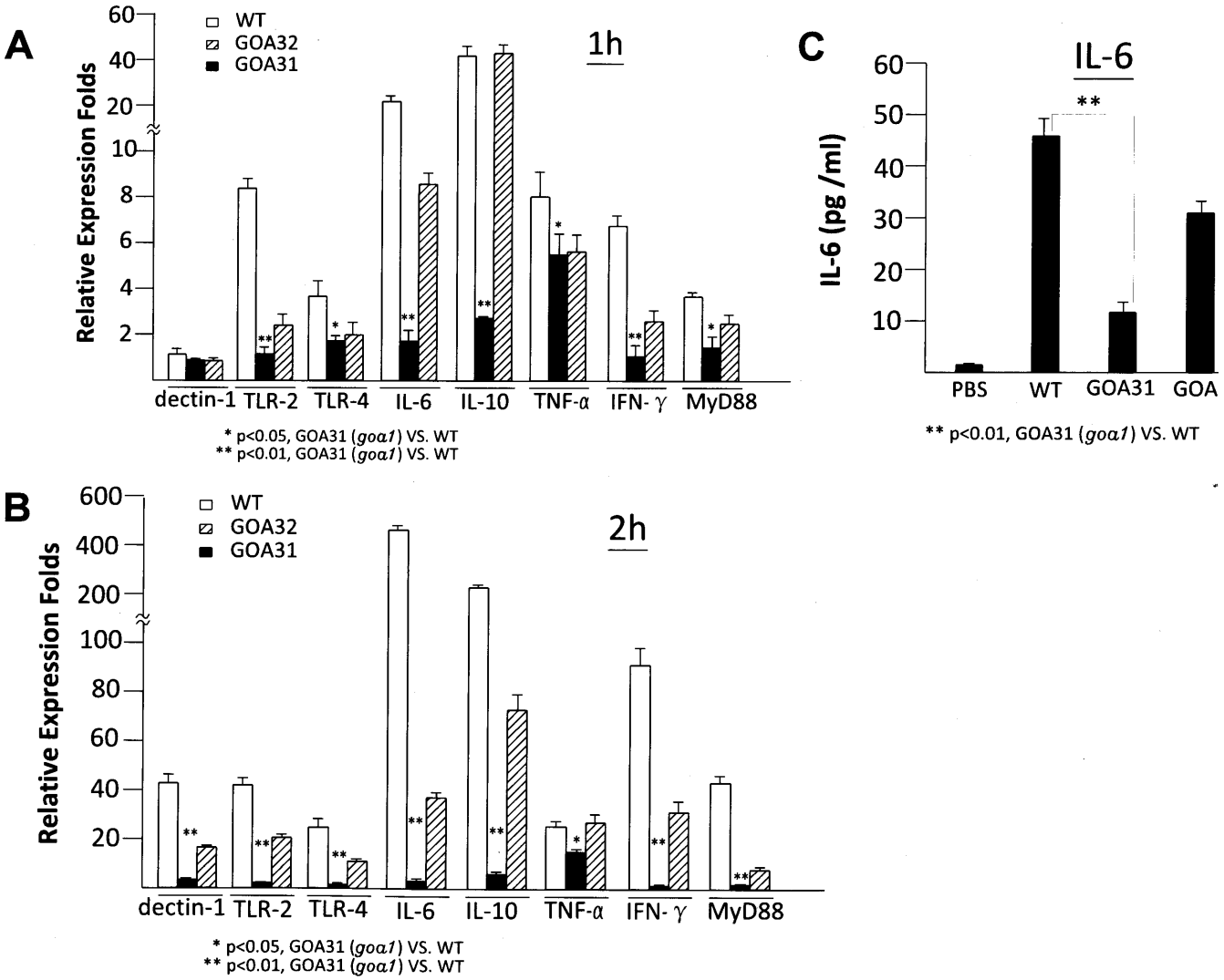

Figure 3.

Expression (qPCR) of cell surface receptors and cytokines of macrophages infected with strains of *C. albicans* at 1 and 2 h (**A and B**, respectively). The macrophage receptors, Dectin-1, TLR-2 and TLR-4 usually synergize to trigger downstream cytokine and adaptor protein expression after binding to ligands such as β-glucan, phospholipomannan and *O*-linked mannosyl residues. Less expression of receptors occurred in co-culture with *goa1*Δ, while the cytokines, IL-6, IL-10, IFN-γ and adaptor protein MyD88 were significantly decreased at 1h. **(C)** The concentration of IL-6 was also determined by ELASA in the supernatants of RAW264.7 cells infected with all yeast strains for 3h to verify the qPCR expression profile. (*P< 0.05; **P< 0.01).

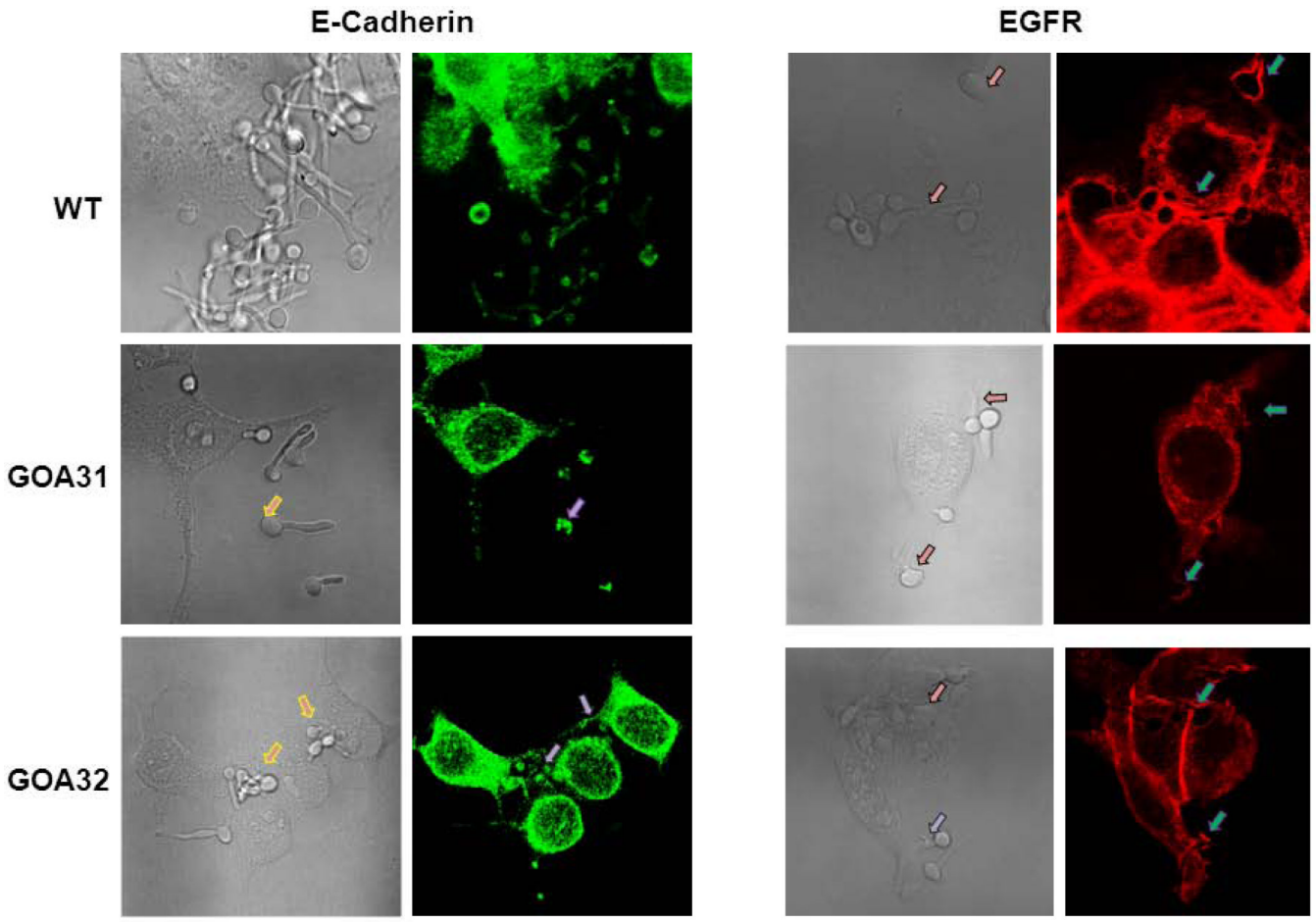

Figure 4.

Confocal microscopic images of the FaDu epithelial cell line E-Cadherin and EGFR. Yeast cells were incubated with FaDu cells for 90 min in MEM Earl's salts plus 10% FBS, then stained with either the anti-ECadherin antibody conjugated with Alexa 488 (left panel) or anti-EGFR antibody conjugated with Alexa 555 (right panel). Images (left panel of each stain) are compared to bright-field images (right panel). The *goa1*Δ null was poorly or partially stained with both antibodies compared to control strains. The arrows indicate the accumulation of fluorescent stain around the hyphae of control strains but not the *goa1*Δ (GOA31).

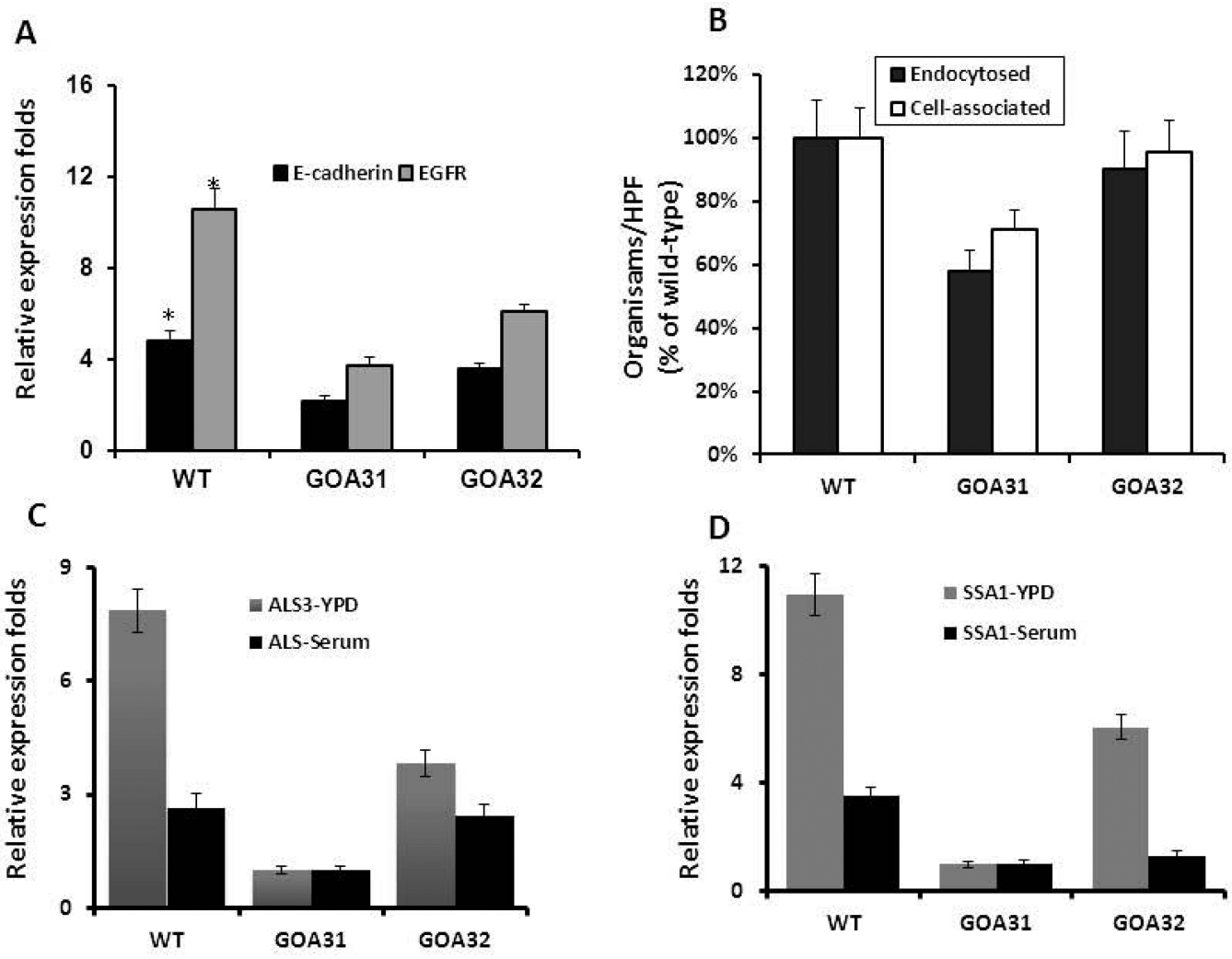

Figure 5.

(A) Expression levels of E-Cadherin and EGFR in FaDu epithelial cells are shown by real-time PCR after infection with each strain post-1h. The endocytosis and cell associated strains are shown after an incubation of 3 h. **(B)** Data are presented for each strain as the percentage of endocytosed or cell-associated cells per 200 epithelial cells. The expression of the *Candida albicans* cell adhesins *ALS3* **(C)** and *SSA1* **(D)** was significantly lower than control strains in both YPD and serum broth.

Table 1

Primer sets used in this study

Gene	Primers
GAPDH (Mouse, NM_008084.2)	Forward: CTTTGTCAAGCTCATTTCCTGG Reverse: TCTTGCTCAGTGTCCTTGC
Dectin-1 (Mouse, AF262985.1)	Forward: TGCAGTGGGTTTAGGAATCC Reverse: GGGCTTGTGGTTCTCTTTATTTC
TLR-2 (Mouse, AF165189.1)	Forward: ACTGTGTTCGTGCTTTCTGAG Reverse: ATGGCTTTCCTCTCAATGGG
TLR-4 (Mouse, AF185285.1)	Forward: GAGGACTGGGTGAGAAATGAG Reverse: GTAGTGAAGGCAGAGGTGAAAG
IL-6 (Mouse, NM_031168.1)	Forward: CAAAGCCAGAGTCCTTCAGAG Reverse: GTCCTTAGCCACTCCTTCTG
IL-10 (Mouse, NM_010548.2)	Forward: AGCCGGGAAGACAATAACTG Reverse: GGAGTCGGTTAGCAGTATGTTG
IFN-γ (Mouse, NM_008337.3)	Forward: CTTTGGACCCTCTGACTTGAG Reverse: TCAATGACTGTGCCGTGG
TNF-α (Mouse, NM_013693.2)	Forward: CTTCTGTCTACTGAACTTCGGG Reverse: CAGGCTTGTCACTCGAA TTTTG
MyD88 (Mouse, NM_010851.2)	Forward: AACAAAGGAACTGGGAGGC Reverse: GTCTGTTCTAGTTGCCGGATC
GAPDH (Human, NM_002046)	Forward: ACATCGCTCAGACACCATG Reverse: TGTAGTTGAGGTCAATGAAGGG
E-Cadherin (Human, NM_004360.3)	Forward: CCCAATACATCTCCCTTCACAG Reverse: CCACCTCTAAGGCCATCTTTG
EGFR (Human, NM_005228.3)	Forward: AAGCCATATGACGGAATCCC Reverse: GGAACTTTGGGCGACTATCTG
18S RNA (*C. albicans*)	Forward: CGCAAGGCTGAAACTTAAAGG Reverse: AGCAGACAAATCACTCCACC
HSP70 (SSA1) (*C. albicans*, orf19. 4980)	Forward: ATTGCTGAAGGTTATTTGGGTTC Reverse: GGTGGCTTGTCTTTGAGAATC
ALS3 (*C. albicans*, orf19. 1816)	Forward: GGAATGCTGTTTTGGGTTGG Reverse: CACCATGAGCAGTCAAATCAAC

Table 2

Summary of down regulated genes encoding cell wall functions in *goa1Δ*

Gene	Fold	Function
Cell wall biogenesis/integrity (n=40)		
orf19.1160, orf19.5412,	0.28-0.29	fungal-type cell wall organization
orf19.719	0.37	
orf19.4984	0.47	chitin catabolic process
orf19.1196	0.44	role in MAPKKK cascade involved in cell wall biogenesis
Orf19.4581	0.48	glycosylphosphatidylinositol-alpha 1,4 mannosyltransferase I
ADA2	0.24	transcriptional coactivator; cell wall integrity role
CHK1	0.21	histidine kinase, regulates cell wall biosynthesis
CHT1,3	0.39, 0.27	chitinase
CRH11	0.33	GPI-anchored cell wall transglycosylase
ECM1,4,7,15,17,21,331	0.16-0.48	cell wall organization or biogenesis
ENG1	0.43	endo-1,3-beta-glucanase
FKS1	0.49	beta-1,3-glucan synthase subunit
MNN4, orf19.5557	0.27-0.49	mannosyltransferase
MNT4	0.45	mannosyltransferase
PHR1,3	0.46, 0.20	beta-1,3-glucanosyltransferase
PIR1	0.34	1,3-beta-glucan-linked structural cell wall protein
IRS4	0.28	roles in cell wall integrity
KIC1	0.47	Ste20p kinases; in RAM cell wall integrity signaling network
PGA10,31,52	0.25-0.47	GPI anchored cell surface protein
SLK19	0.39	alkaline-induced membrane protein; affects cell aggregation
SMI1	0.48	cell wall biosynthesis protein
SUR7	0.38	required for normal cell wall, plasma membrane
XOG1	0.18	exo-1,3-beta-glucanase
BGL22	0.21	glucanase; induced during cell wall regeneration
BMT3	0.08	beta-mannosyltransferase
EXG2	0.44	GPI-anchored cell wall protein
Orf19.5070	0.42	cell-wall mannoproteins
VPS28	0.49	proteolytic activation of Rim101p, role in echinocandin sensitivity
Cell membrane associated genes (n=6)		
PLB5	0.42	GPI-linked phospholipase B
YPT31	0.50	required for resistance to toxic ergostero analog
ERG27	0.42	3-Keto sterol reductase of ergosterol biosynthesis
Orf19.3226	0.28	intracellular sterol transport
orf19.4096	0.06	acylglycerophosphocholine O-acyltransferase
orf19.7547	0.27	phosphatidylinositol-3-phosphate binding
Adhesin/antigenic genes (n=26)		
ALS1, 2,3,4,5,6,7	0.08-0.49	ALS family protein, cell-surface glycoprotein, adhesion

Gene	Fold	Function
IFF4,5,8,11	0.11-0.43	GPI-anchored protein , adhesion-like protein
HSP70	0.15	antigenic, role in entry into host cells
HYR3	0.45	adhesion-like protein
orf19.251	0.30	binds human immunoglobulin E
AHP1	0.42	alkyl hydroperoxide reductase; immunogenic in mouse
CSH1	0.38	aldo-keto reductase family member, role in fibronectin adhesion
CYC3	0.50	cytochrome c heme lyase, antigenic cell-wall protein
DDR48	0.05	immunogenic stress-associated protein
MET15, 6	0.04, 0.23	antigenic during murine or human systemic infection
PGA62	0.44	adhesin-like cell wall protein
RBR3	0.37	cell wall adhesin-like protein
PST2	0.07	NADH:quinone oxidoreductase, immunogenic in mice
SAP10, 9	0.22, 0.24	secreted aspartyl protease; roles in adhesion
XYL2	0.16	D-xylulose reductase; immunogenic in mice
Yeast-hyphal morphological transition genes (n= 14)		
CEK1	0.44	ERK-family protein kinase, yeast-hyphal switching
CHO1	0.33	phosphatidylserine synthase, required for filamentous growth
CSP37	0.25	hyphal cell wall protein
DFG5	0.28	N-linked mannoprotein, role in hyphal growth
GPH1	0.28	glycogen phosphorylase, localizes to cell surface of hyphae
MID1	0.38	high affinity calcium uptake system, role in thigmotropism
PHO85,111	0.50,0.50	acid phosphatase, negatively regulated by Rim101p
RBT5	0.09	GPI-anchored cell wall protein
RHD3	0.32	GPI-anchored cell wall protein; yeast-associated protein
RVS167	0.40	involved in endocytosis; hyphal growth required
SAM2	0.50	S-adenosylmethionine synthetase, localizes to surface of hyphal cells
YDC1	0.21	Mob2p-dependent hyphal regulatio
SNZ1	0.12	induced on yeast to hyphal switch