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The Hyphal-Associated Adhesin and Invasin Als3 of *Candida albicans* Mediates Iron Acquisition from Host Ferritin

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**Abstract**

Iron sequestration by host iron-binding proteins is an important mechanism of resistance to microbial infections. Inside oral epithelial cells, iron is stored within ferritin, and is therefore not usually accessible to pathogenic microbes. We observed that the ferritin concentration within oral epithelial cells was directly related to their susceptibility to damage by the human pathogenic fungus *Candida albicans*. Thus, we hypothesized that host ferritin is used as an iron source by this organism. We found that *C. albicans* was able to grow on agar at physiological pH with ferritin as the sole source of iron, while the baker's yeast *Saccharomyces cerevisiae* could not. A screen of *C. albicans* mutants lacking components of each of the three known iron acquisition systems revealed that only the reductive pathway is involved in iron utilization from ferritin by this fungus. Additionally, *C. albicans* hyphae, but not yeast cells, bound ferritin, and this binding was crucial for iron acquisition from ferritin. Transcriptional profiling of wild-type and hyphal-defective *C. albicans* strains suggested that the *C. albicans* invasin-like protein Als3 is required for ferritin binding. Hyphae of an *Als3* null mutant had a strongly reduced ability to bind ferritin and these mutant cells grew poorly on agar plates with ferritin as the sole source of iron. Heterologous expression of Als3, but not Als1 or Als5, two closely related members of the Als protein family, allowed *S. cerevisiae* to bind ferritin. Immunocytochemical localization of ferritin in epithelial cells infected with *C. albicans* showed ferritin surrounding invading hyphae of the wild-type, but not the *Als3* mutant strain. This mutant was also unable to damage epithelial cells in vitro. Therefore, *C. albicans* can exploit iron from ferritin via morphology dependent binding through Als3, suggesting that this single protein has multiple virulence attributes.

**Introduction**

Iron is an essential element for virtually all organisms, ranging from microbes to multicellular animals. Higher organisms can sequester iron using high-affinity iron-binding molecules, so that it is unavailable to microorganisms. Iron sequestration provides a natural resistance to infections which has been described as “nutritional immunity” [1].

Successful microbial pathogens have developed multiple iron acquisition and uptake systems (reviewed in [2,3]). These systems include enzymes for reduction and oxidation of iron ions (Fe²⁺ or Fe³⁺), high-affinity permeases for iron transport, chelators ( siderophores) and uptake systems for siderophores. In the human body, the majority of iron is bound to iron-containing proteins with physiological functions (for example heme proteins such as hemoglobin), iron-binding transport proteins (transferrin), antimicrobial proteins ( lactoferrin), or cellular iron storage proteins (ferritin). With the exception of ferritin, each of these proteins has been reported to serve as an iron source for some pathogenic microbes. These iron sources are exploited via direct binding, degradation, and/or uptake [4–13].

In mammalian cells, extracellular ferric iron is bound by apotransferrin (transferrin without iron). The binding of apotransferrin to two ferric iron molecules (holotransferrin, hTF) increases by two-fold its affinity for the transferrin receptor (TFR) present on the surface of virtually all mammalian cells. Following endocytosis of the hTF-TFR complex into the early endosome, acidification to low pH (pH 5.6) results in the release of iron from holotransferrin. The released ferrous iron is then transported to the cytoplasm by the divalent metal transporter (DMT1) and either used for cellular metabolism or stored within ferritin. The resulting apotransferrin is recycled to the cell surface and released at physiological pH (7.4) [14–16].

Ferritin is the main intracellular storage protein for iron (reviewed in [17]), containing approximately 30% of the total human body iron (66% is bound to hemoglobin). Ferritin consist of

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Iron is an essential nutrient for all microbes. Many human pathogenic microbes have developed sophisticated strategies to acquire iron from the host as most compartments in the body contain little free iron. For example, in oral epithelial cells intracellular iron is bound to ferritin, a protein that is highly resistant to microbial attack. In fact, no microorganism has so far been shown to directly exploit ferritin as an iron source during interaction with host cells. This study demonstrates that the pathogenic fungus Candida albicans can use ferritin as the sole source of iron. Most intriguingly, C. albicans binds ferritin via a receptor that is only exposed on invasive hyphae. This receptor is Als3, which is a member of the Als-protein family. Als3 was previously demonstrated to be an adhesin with invasin-like properties. Mutants lacking Als3 failed to bind ferritin, grew poorly with ferritin as an iron source and were unable to damage epithelial cells. Strains of the baker’s yeast expressing C. albicans Als3, but not two closely related proteins, Als1 or Als5, were able to bind ferritin. Therefore, C. albicans uses an additional morphology specific and unique iron uptake strategy based on ferritin while invading into host cells where ferritin is located.

C. albicans can grow in almost all body sites and organs, indicating an astonishing metabolic flexibility, a high level of stress resistance and effective immune evasion strategies. One of the key features of C. albicans is its ability to grow in different morphological forms—either as ovoid yeast, a filamentous hyphal form or as pseudohyphae [30]. Although the yeast form appears to be important for dissemination [31], the hyphal form is of crucial importance for cell and tissue invasion [32–34]. Furthermore, genes known or proposed to be associated with adhesion, invasion, extracellular hydrolytic activity, detoxification or as yet unknown functions (HWP1, ALS3, SLP1–6, SOD5, HYR1, ECE1) are co-regulated with the yeast-to-hyphal transition [35–40]. Both cellular morphology and expression of hyphal associated genes are tightly regulated by a network of signal transduction pathways (including MAP kinase, cAMP and Rim101 pathways [30,41]) and transcriptional activators and repressors such as Efg1, Tec1, Bcr1, Tup1 and Nrg1 [42–46].

C. albicans adaptation to the host environment is also reliant on a large number of genes associated with iron acquisition [47]. These genes contribute to the three known iron acquisition systems of C. albicans: (1) Uptake and utilization of iron from hemoglobin is mediated by Rbt5 and Hmx1 [13,48,49]. In vitro data have shown that Rbt5 is a hemoglobin receptor that binds hemoglobin on the surface. This binding seems to induce expression of HMX1, which encodes a heme oxygenase. This activity is essential for iron utilization from heme [49]. (2) Iron in siderophores is taken up via the siderophore transporter, Sit1 [50,51]. C. albicans siderophore production had been demonstrated by biochemical assays in earlier studies [52,53]. However, in contrast to Aspergillus fumigatus [54], genes encoding factors of a possible siderophore production pathway have not been discovered in the C. albicans genome [47]. Nevertheless, Sit1 can mediate uptake of a range of heterologous siderophores from other organisms and other iron complexes [50,55–57]. (3) To use free iron from the environment, iron from transferrin, and possibly iron from other so far unknown sources, C. albicans uses the reductive uptake system. This system is located in the plasma membrane and has three components. The first component consists of ferric reductases. At least two surface ferric reductases, which are able to reduce insoluble extracellular ferric (Fe3+) ions into soluble Fe2+ ions, have been described [7,58,59]. In addition thirteen homologous genes, putatively encoding other ferric reductases have been identified in the C. albicans genome (http://www.Candidagenome.org). The second component consists of multicopper oxidase. Reduced ferrous iron generated by surface reductase activity can be toxic due to spontaneous generation of free radicals. However, Fe2+ can also be oxidized to Fe3+ by multicopper oxidase activity and thus preventing the production of toxic free radicals [60,61]. The C. albicans genome contains five putative multicopper oxidase genes [62]. Due to the copper requirement of the oxidase activity, the intracellular copper transporter Ccc2 is essential for this reductive pathway [63]. The third component consists of iron permeases. These form a protein complex with multicopper oxidases and transport Fe3+ into the cell. C. albicans has two iron permeases that are encoded by two highly homologous genes. The high-affinity iron permease gene, FTR1 is induced by iron deprivation and the low-affinity iron permease gene, FTR2 is induced when higher levels of iron are available [64].

All three iron acquisition systems appear to be independent from each other and so far only Ftr1 has been shown to be crucial for C. albicans virulence in an experimental animal model of infection [64]. However, it is unclear which iron sources are used during the different types of C. albicans infection and within different anatomical sites. Recent in vitro and in vivo transcriptional profiling experiments have shown that C. albicans gene expression is tissue specific [33,34,65]. Since the relative proportion of iron-
containing proteins differs among the different anatomical sites, we propose that usage of iron by *C. albicans* is niche specific.

Within the oral cavity, extracellular iron is bound mostly to lactoferrin in saliva and intracellular iron is stored in ferritin. However, oral infections by *C. albicans* are frequent, suggesting that *C. albicans* must be able to exploit the host iron resources of the oral cavity. We observed that genes encoding the high-affinity reductive iron uptake system of *C. albicans* are up-regulated during oral infections in patients [33]. Also, *C. albicans* causes greater damage to oral epithelial cells that contain a high concentration of ferritin (this study). Therefore, we hypothesized that host ferritin may be used as an iron source by this organism. Here we show that *C. albicans* can utilize iron from ferritin via morphology dependent binding through the adhesin and invasin Als3, suggesting that this single protein has multiple virulence attributes.

**Results**

**The Ferritin Content of Epithelial Cells Influences the Extent of Cellular Damage Caused by *C. albicans***

*C. albicans* can attach to and proliferate on oral epithelial tissue and can invade and damage epithelial cells [66]. To elucidate which iron sources are exploited during growth on and invasion of oral epithelial cells and to determine how the availability of iron influences fungal-host cell interactions, we incubated oral epithelial cell monolayers in the presence of additional free iron or the iron chelator bathophenanthrolindisulphonic acid (BPS).

This chelator sequesters extracellular, but not intracellular iron [67]. Through immunocytochemical localization of ferritin within epithelial cells, we found that addition of BPS caused a dramatic decrease in cellular ferritin within 24 hours of incubation (Figure 1A), in comparison to non-treated cells (Figure 1B). In contrast, addition of free iron to the medium increased the concentration of ferritin within the same time period (Figure 1C).

The treatment with additional iron or the iron chelator itself did not cause cell damage, as monitored by measuring the release of epithelial lactate dehydrogenase (LDH) into the supernatant (not shown).

Next, ferritin enriched or depleted epithelial monolayers were incubated for 8 h with *C. albicans* in normal cell culture medium (serum-free RPMI1640) and cell damage caused by *C. albicans* was monitored by LDH release. The epithelial monolayers depleted of ferritin were significantly protected from damage in comparison to untreated monolayers (control) (Figure 1D). In contrast, ferritin

![Figure 1](https://example.com/figure1.png)
enriched epithelial cells were significantly and dose dependently more susceptible to damage caused by \textit{C. albicans} (Figure 1D). These observations suggested that the ferritin content of epithelial cells directly correlates with cell damage and opened up the possibility that \textit{C. albicans} can use ferritin as an iron source.

**Depletion of Ferritin, but not Ferritin Saturation Influences Invasion of Epithelial Cells by \textit{C. albicans}**

To clarify whether the observed increased or decreased cytotoxicity was due to either reduced or increased invasion of epithelial cells by \textit{C. albicans}, we quantified invasion (after 3 h of co-incubation) in iron depleted versus iron saturated epithelial cells. Invasion of \textit{C. albicans} into iron depleted epithelial cells was drastically reduced (Figure 2). It is known that \textit{C. albicans} must invade oral epithelial cells to cause epithelial cell damage [68]. Therefore, the decreased epithelial cell invasion likely contributed to the reduced epithelial cell damage caused by iron depletion. In contrast, iron saturated epithelial cells were invaded at the same proportion as compared to untreated cells (Figure 2), even though \textit{C. albicans} caused much more damage to these cells. These results suggest that the iron content of epithelial cell influences their susceptibility to damage by \textit{C. albicans}, a mechanism that is at least partially independent of the extent of epithelial cell invasion.

**\textit{C. albicans} Can Use Ferritin as the Sole Source of Iron \textit{in vitro}**

One explanation for the increased susceptibility of iron saturated epithelial cells to damage by \textit{C. albicans} is that the organism uses epithelial cell ferritin as an iron source and is thereby able to produce more cytotoxic factors. To test whether \textit{C. albicans} can use ferritin as an iron source \textit{in vitro}, we incubated fungal cells on agar with ferritin as the sole iron source. By addition of BPS to the medium, we were able to remove any residual iron from the agar, medium or plastic surfaces. Only the addition of an external iron source allowed fungal growth under these conditions. Moreover, to minimize possible iron contamination of the ferritin solutions (not shown), we passed the ferritin through a column (Microcon YM-100, see Material and Methods) and washed it once with 5 mM HEPES buffer (pH 7.4) prior to use. Addition of free ferric iron, hemoglobin or ferritin to the agar promoted the growth of \textit{C. albicans} at pH 7.4 (Figure 3A). In contrast, the baker’s yeast \textit{Saccharomyces cerevisiae}, known to be unable to grow with hemoglobin as the sole source of iron [13], only grew after addition of free iron to the medium (Figure 3A). However, \textit{S. cerevisiae} was able to grow with ferritin when the initial pH of the medium was calibrated to pH 5.0 (not shown). This result suggested that the external pH of the medium influenced the bio-availability of iron from ferritin.

**The Use of Ferritin as the Sole Source of Iron \textit{in vitro} Requires the Reductive Pathway and is Mediated by Acidification of the Medium**

The ferritin protein shell is unstable at acidic pH [18]. Therefore, our finding that \textit{S. cerevisiae} can utilize iron from ferritin at acidic, but not alkaline pH, suggested the possibility that \textit{C. albicans} is able to release iron from this protein by active

![Figure 2. Invasion of ferritin depleted or enriched epithelial cells by \textit{C. albicans}. Approximately $10^5$ iron starved \textit{C. albicans} cells (SC5314) were co-incubated with ferritin depleted (BPS), ferritin enriched (Fe50) or non-treated (Control) epithelial cells for 3 h. After fixation the samples were differentially stained and analyzed under the fluorescence microscope. The experiment was performed three times in duplicate. *, significant difference compared to non-treated epithelial cells (control) (p<0.001). doi:10.1371/journal.ppat.1000217.g002](#)

![Figure 3. Usage of ferritin by \textit{C. albicans} requires the reductive pathway and is mediated by acidification of the medium. (A) SD agar plates were adjusted to pH 7.4 with 25 mM HEPES buffer and incubated for 3 days at 37°C under 5% CO$_2$ (Ca, \textit{C. albicans} SC5314) or 30°C without CO$_2$ (Sc, \textit{S. cerevisiae} ATCC9763). Iron indicates 50 µM iron sulphate. Ferritin indicates 20 µg/ml ferritin. Hemoglobin indicates 20 µg/ml hemoglobin. (B) \textit{C. albicans} wild-type (SC5314) cells were spotted on YNB agar with the addition of either glucose (SD) or casamino acids as a carbon source and buffered with 25 or 200 mM HEPES. BPS (iron chelator) was used to remove free iron from the media. The growth of \textit{C. albicans} strains and \textit{S. cerevisiae} on agar with different iron sources was repeated at least 3 times. doi:10.1371/journal.ppat.1000217.g003](#)
acidification of the medium. In fact, *C. albicans* was able to acidify a medium buffered with 25 mM HEPES (initial pH 7.4) during incubation with ferritin as sole source of iron as monitored by the pH indicator bromocresol green (Figure S1). To determine whether ferritin utilization is dependent on fungal-driven acidification, we substituted the glucose in the medium for casamino acids. This mixture of amino acids can be used as a carbon source by yeasts and avoids the acidification associated with glucose use [69,70]. Furthermore, we stabilized the buffering capacity of the medium by the addition of HEPES buffer (pH 7.4) with increasing concentrations. As shown in Figure 3B, decreasing the capacity to acidify the medium, reduced the ability of *C. albicans* to grow with ferritin as a sole source of iron.

Next, we sought to determine which of the three known iron uptake systems of *C. albicans* are involved in iron acquisition from ferritin. Mutants lacking key genes of each iron acquisition system were screened for growth on ferritin agar plates. A mutant lacking the high-affinity permease *Ftr1* was able to grow with free iron, hemoglobin, but not with ferritin as the sole iron source (Figure 3A; Table 1). Similarly, a mutant, lacking the copper transporter *Ccc2*, which is also essential for the reductive pathway, did not grow on ferritin plates (Figure S2; Table 1). In contrast to *S. cerevisiae*, *Aft1* and *Ace2* mutants did not grow on ferritin plates even when the initial pH was reduced to 5.0 (not shown). As expected, the *Aft1*+*Ftr1* and *Ace2*+*Cc2* re-integrant strains grew similarly to the wild-type strain in the presence of ferritin (Figure S2). These observations suggest that the reductive pathway is essential for *C. albicans* to acquire iron from ferritin.

The *Asi1* and *Aft5* mutants grew normally when ferritin was the sole iron source, indicating that *C. albicans* utilization of iron from ferritin is independent of the siderophore and hemoglobin uptake systems (Figure S2; Table 1).

We also investigated the possibility that aspartic proteases secreted by *C. albicans* could break down ferritin and release iron. The *Asap1-3* and *Asap4-6* triple-mutants grew similarly to wild-type cells on ferritin plates, suggesting that secreted aspartic proteases of *C. albicans* are not involved in liberating iron from ferritin (Figure S2; Table 1).

### Hyphal, but not Yeast Cells of *C. albicans* Can Bind Ferritin

Pathogenic microbes frequently utilize iron from host proteins by binding these molecules via specific receptors [4–8,12,13,71]. Since our data showed that *C. albicans* can use ferritin as a sole source of iron, we investigated whether *C. albicans* can bind ferritin on its surface.

*C. albicans* cells preincubated in iron limited medium (LIM0) were co-incubated with ferritin and then rinsed extensively. The ferritin on its surface.

The finding that ferritin was not bound by either yeast cells or the mother cells of hyphae suggested that ferritin binding was specific to *C. albicans* hyphae. To test this hypothesis further, we investigated the ferritin binding of *C. albicans* *ars1* and *Ahp1/efg1* mutants that were unable to form hyphae, and did not express hyphal-specific genes [72,73]. Both mutants were unable to bind ferritin (Figure 4A and 4B). Next, we tested the ferritin binding capacity of a *Ahg1* mutant, which forms pseudohyphae rather than true hyphae, but still expresses hyphal-specific genes [74]. When grown under hyphal-inducing conditions (RPMI 1640, 37°C with 5% CO₂), the *Ahg1* mutant bound ferritin even though it did not form true hyphae (Figure 4A and 4B). These results suggest that the product of one or more hyphal specific genes is essential for *C. albicans* to bind ferritin.

To uncover which hyphal-associated activities are involved in ferritin binding, wild-type hyphae were killed with thimerosal or UV light and tested for ferritin binding. Cells killed with thimerosal still bound ferritin; whereas cells killed with UV light did not (Figure S3A). When untreated wild-type cells were mixed with 50% UV-killed cells, we observed 49.06%±2.27% ferritin binding. These data demonstrate that cell viability is not necessary for ferritin binding and that the ferritin receptor on the cell surface is inactivated by UV treatment. We also investigated whether iron availability influenced the extent of ferritin binding of wild-type hyphae. Cells grown under iron limiting conditions or in the presence of excess iron bound ferritin similarly (Figure S3B). Also, *C. albicans* hyphae were able to bind ferritin and apoferitin (a ferritin shell without iron) with similar efficiency (not shown) indicating that iron molecules within the ferritin shell were dispensable for binding of ferritin. Thus, these data indicated that the binding of ferritin by *C. albicans* is morphology associated, but not iron-regulated.

### Transcriptional Profiling of *C. albicans* Cells Binding Ferritin Identifies Putative Genes Necessary for Ferritin Binding

Transcriptional profiling was used to identify genes encoding putative ferritin receptors. We incubated a wild-type strain (true hyphae and ferritin binding), the *Ahg1* mutant (yeast or pseudohyphae and ferritin binding) and *ars1* (no hyphae and no ferritin binding) under hyphal-inducing conditions (RPMI

| Table 1. Growth of different strains on ferritin agar plates. |
|---------------------------------------------------------------|
| Wild-type | Reductive pathway | Siderophore and hemoglobin receptors | Aspartic proteases |
| CAF2-1 | CAF2-1 | CAF2-1 | CAF2-1 | CAF2-1 |
| SD | + | + | + | + | + | + | + |
| SD+BPS | – | – | – | – | – |
| SD+BPS+5 μg/ml ferritin | + | – | – | + | – | + | + |

SD agar was buffered using 100 mM HEPES (pH 7.4). BPS, iron chelator. All plates were incubated for 3 days at 37°C under 5% CO₂. See Figure S1 for details.

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medium, 37°C with 5% CO2) and in the presence of ferritin. After 1.5 h, the RNA from all three strains was isolated, labeled and hybridized to C. albicans microarrays. Microarray data from four independent experiments were analyzed. We reasoned that candidate genes encoding putative ferritin receptors should be up-regulated in wild-type and Δhgc1 cells, but should be unaltered or down-regulated in the Δras1 mutant (Figure 5). A total of 22 genes were identified with such an expression profile (Figure 5).

Expression data shown in Table 2 indicate the genes that were up-regulated in wild-type cells but not in Δras1 mutant cells. Three of these genes were known to encode hyphal-specific proteins that are cell surface localized as would be expected for a receptor protein. Consequently, these three genes were further investigated.

Deletion of ALS3 Precludes Ferritin Binding

The three genes encoding cell surface localized and hyphal-specific proteins were ECE1, HYR1 and ALS3. ECE1 (Extent of Cell Elongation) is a hyphal-specific gene with yet unknown functions. ECE1 expression increases during elongation of the hyphal cell. This gene encodes a predicted cell membrane protein and the corresponding null mutant displays no obvious altered phenotype [40]. HYR1 (HYphally Regulated) encodes a GPI-anchored protein that is predicted to be cell wall localized and is of unknown function [39]. Finally, ALS3 (Agglutinin-Like Sequence) encodes a hyphal-specific cell wall protein which belongs to a family of adhesins [Als family] [75] and plays a crucial role in epithelial and endothelial adhesion and invasion [32].

The corresponding homozygous null mutants were tested for ferritin binding. Both, the Δece1 and the Δhyr1 mutants bound ferritin similarly to the wild-type strain (Figure 6A and B). In contrast, ferritin binding of the Δals3 mutant was dramatically reduced (Figures 6 and 7). This defect in ferritin binding was restored when a wild-type copy of ALS3 was reintegrated into the Δals3 mutant (Figures 6 and 7).

These results suggested that Als3 plays a crucial role in ferritin binding and may in fact be the hyphal-specific ferritin receptor.

Upstream Regulators of ALS3 are Required for Ferritin Binding

If Als3 is a ferritin receptor, one would expect that mutants lacking factors that govern ALS3 expression would also have an altered capacity to bind ferritin. Therefore, we tested two mutants that lacked ALS3 transcriptional regulators. BCR1 encodes a transcription factor which regulates the expression of certain hyphal-specific genes, including ALS3 [76]. Furthermore, expression of BCR1 itself depends on Tec1 [44]. Figure 6C shows that the presence of both transcriptional factors, Tec1 and Bcr1, is necessary for C. albicans cells to bind ferritin. These data reinforce the view that Als3 plays a key role in the capacity of C. albicans to bind ferritin.

Binding is Necessary for Iron Acquisition from Ferritin

To determine whether ferritin binding is necessary for the utilization of iron from this protein, we tested the growth of the Δals3 mutant with ferritin as the sole iron source.
mutant grew very poorly on agar plates (pH 7.4) with ferritin as the sole source of iron (Figure 8). This reconstitution of one copy of the gene \( \Delta \text{hgc1}+\Delta \text{als3} \) re-integrant strain, improved growth, although not to wild-type levels (Figure 8). Growth of the \( \Delta \text{als3} \) mutant in media with low iron content was not reduced, indicating that uptake of free iron is normal in the \( \Delta \text{als3} \) mutant (not shown). Therefore, Als3 is required for \( C. \text{albicans} \) hyphae to both bind and utilize ferritin as a source of iron.

Moreover, a mutant unable to form hyphae (\( \Delta \text{ras1} \)) and thus unable to bind ferritin was also tested for growth on ferritin plates. As expected, \( \Delta \text{ras1} \) displayed a reduced ability to grow with ferritin as the sole source of iron (Figure S4). This result reinforces the key role of hyphal development and the hyphal associated expression of ALS3 in the ability of \( C. \text{albicans} \) to obtain iron from ferritin.

Als3 is a Ferritin Receptor

To elucidate whether Als3 itself can bind ferritin without an additional \( C. \text{albicans} \) surface factor, we tested the ferritin binding capacity of a strain of \( S. \text{cerevisiae} \) that expressed \( C. \text{albicans} \) ALS3 [77]. Because ALS3 is a member of a large gene family encoding similar proteins, we also analyzed two additional \( S. \text{cerevisiae} \) strains that expressed ALS1 or ALS5, two closely related ALS genes. The strain that expressed ALS3 strongly bound ferritin, whereas the strains that expressed ALS1 or ALS5 did not (Figure 9).

Invading \( C. \text{albicans} \) Hyphae Bind Ferritin from Epithelial Cells during Infection

Next we investigated whether ferritin binding via Als3 occurs when \( C. \text{albicans} \) interacts with host cells. Oral epithelial cells were loaded with iron and then incubated with wild-type \( C. \text{albicans} \), the \( \Delta \text{als3} \) mutant, or the \( \Delta \text{als3}+\Delta \text{ALS3} \) re-integrant strain for 6 h.

To visualize ferritin molecules on cellular surfaces and to investigate whether the location of fungal cells had an influence on ferritin binding, we used an immunofluorescence approach with differential staining, which enabled us to discriminate between hyphae located on the epithelial cell surface and hyphae that had invaded into the epithelial cells (Figure 10 columns 1, 2 and 4). In addition, we used an anti-ferritin antibody to localize ferritin.

Table 2. Genes up-regulated in wild-type and \( \Delta \text{hgc1} \) cells, but unaltered or down-regulated in the \( \Delta \text{ras1} \) mutant (Figure 5).

| Gene name | Fold up-regulated in wild-type cells | Description |
|-----------|-------------------------------------|-------------|
| ECE1      | 22.0                                | cell elongation protein |
| UME6      | 6.6                                 | transcription factor |
| PUT2      | 5.3                                 | 1-pyrroline-5-carboxylate dehydrogenase (by homology) |
| ALS3      | 5.0                                 | agglutinin like protein |
| orf19.4805| 5.0                                 | unknown function |
| FAS2      | 4.4                                 | fatty-acyl-CoA synthase (internal fragment) |
| ADO1      | 3.8                                 | adenosine kinase (by homology) |
| orf19.2210| 3.4                                 | unknown function |
| FAS1      | 3.3                                 | fatty-acyl-CoA synthase |
| ABC1      | 3.2                                 | acyl-CoA binding (by homology) |
| ACC1      | 3.2                                 | acetyl-CoA carboxylase (by homology) |
| ERG25     | 3.2                                 | C-4 methylsterol oxidase (by homology) |
| orf19.4468| 3.0                                 | succinate dehydrogenase (by homology) |
| orf19.5147| 2.9                                 | unknown function |
| UAP1      | 2.8                                 | UDP-N-acetylglucosamine pyrophosphorylase |
| orf19.801 | 2.8                                 | unknown function |
| FAS2      | 2.7                                 | fatty-acyl-CoA synthase (3-prime end) |
| HYR1      | 2.6                                 | hyphally regulated protein |
| orf19.5126| 2.6                                 | unknown function |
| RPS9B     | 2.6                                 | ribosomal protein (by homology) |
| orf19.2650| 2.6                                 | mitochondrial ribosomal protein (by homology) |
| orf19.1186| 2.5                                 | unknown function |

Genes encoding hyphal surface proteins are in bold. Given data for wild-type cells were similar to data obtained for \( \Delta \text{hgc1} \) cells (not shown).

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C. \text{albicans} Mutants Lacking Genes Essential for Iron Utilization from Ferritin are Unable to Damage Epithelial Cells

If binding to ferritin and utilizing host iron are important for \( C. \text{albicans} \) to cause an oral infection, one would expect that mutants...
lacking ALS3 or FTR1 would have a reduced potential to cause tissue damage as compared to wild-type cells. To test this prediction, we measured the extent of epithelial cell damage caused by wild-type, \textit{Als3} mutant and \textit{Aftr1} mutant strains of \textit{C. albicans}. We found that the \textit{Als3} and \textit{Aftr1} mutants lost their capacity to damage epithelial cells (Figure 11). In contrast to \textit{Als3} mutant cells, which displayed strongly reduced invasion abilities when co-incubated with epithelial cells for 3 hours (not shown), \textit{Aftr1} mutant cells showed the same invasion rate than the wild-type strain (Figure S5). Although hyphae of this mutant seemed shorter than the wild-type hyphae, there was no morphological differences between \textit{Aftr1} mutant cells on epithelial cells and in RPMI medium alone (control) (not shown).

**Discussion**

Iron availability is a critical factor for all pathogenic microbes and iron excess can accelerate pathogenicity [1,78–80]. We observed that oral epithelial cells enriched in intracellular ferritin were more susceptible to tissue damage by wild-type \textit{C. albicans} and that epithelial cells depleted of ferritin were significantly protected from damage. The reduced damage of iron depleted epithelial cells correlated with reduced invasion abilities when co-incubated with epithelial cells for 3 hours (not shown), \textit{Aftr1} mutant cells showed the same invasion rate than the wild-type strain (Figure S5). Although hyphae of this mutant seemed shorter than the wild-type hyphae, there was no morphological differences between \textit{Aftr1} mutant cells on epithelial cells and in RPMI medium alone (control) (not shown).

It is known that ferritin is an extremely robust and resistant protein. Prior to this study, the only microorganism that has been known to exploit holoferritin as an iron source during interaction with host cells is \textit{N. meningitidis} [21,22]. However, this bacterium is not able to directly utilize iron from ferritin. Instead, it induces degradation of cytosolic ferritin by manipulating the host cellular machinery and thereby utilizes the resultant free cytosolic iron. To our knowledge, no published studies have so far demonstrated direct use of iron from host ferritin. Nevertheless, a number of studies have suggested that certain microbial pathogens can use ferritin as an iron source during \textit{in vitro} growth. For example, \textit{Yersinia pestis} can grow on agar containing hemin, myoglobin, hemoglobin or ferritin [83]. A siderophore produced by \textit{M. tuberculosis} (exochelin) can sequester iron from transferrin, lactoferrin and to a lesser extent from ferritin [86]. \textit{L. monocytogenes} and \textit{Burkholderia cenocepacia} can grow in liquid
medium with ferritin as the sole source of iron [87,88]. However, the microbial mechanisms of iron acquisition from ferritin are unknown and it is not clear whether ferritin from host cells can be used by any of these species. Furthermore, although ferritin seems to be almost indestructible under physiological conditions, iron may be released from ferritin in vitro, especially under condition of low pH. In our hands, even *S. cerevisiae* was able to utilize iron from ferritin under such conditions. Therefore, it is possible that previous observations of the microbial usage of ferritin in vitro were the results of non-physiological conditions.

In contrast to *S. cerevisiae*, *C. albicans* can use ferritin as the sole source of iron in vitro even when the growth medium was buffered at a physiological pH. Which mechanisms and activities are involved in iron acquisition from ferritin?

One possibility is that ferritin is degraded by extracellular proteolytic activity since it is known that *C. albicans* can secrete a family of aspartic proteases (*Saps*) with very broad substrate specificity [89]. However, it appears that extracellular degradation due to fungal proteases is not necessary for growth with ferritin, since mutants lacking the protease genes *SAP1-3* or *SAP4-6* were still able to grow on such medium. Indeed, an earlier study by Ruchel demonstrated that ferritin was the only tested protein which was resistant to proteolysis by Sap2, one of the major secreted proteases of *C. albicans* with an extremely broad substrate specificity [90], supporting the view that proteases are not involved in the ability of *C. albicans* to utilize iron from ferritin.

Since even *S. cerevisiae* was able to grow with ferritin when the pH of the medium was low (pH 5.0), we reasoned that the pH plays a crucial role in the release of iron from ferritin. It is known that ferritin is unstable at acidic pH [18] and that the natural recycling of iron from ferritin occurs in the acidic environment of lysosomes [19,20]. Thus, it may be possible that *C. albicans* actively lowers the pH in its proximate vicinity. In fact, *C. albicans* was able to lower the pH of the

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**Figure 7. Flow cytometric detection of ferritin binding.** *C. albicans* cells were incubated under hyphal-inducing conditions (RPMI, 37°C with 5% CO₂) for 2 h. After 1 h in the presence of 100 μg/ml ferritin, cells were washed and ferritin was stained using indirect immunofluorescence and then analyzed using flow cytometry. (A) wild-type (CAF2-1); (B) Δals3; (C) Δals3+ALS3. Fluorescence data for 10,000 cells of each strain were collected. (D) Binding quantification. The data are expressed as a percentage of the results obtained with the wild-type strain (CAF2-1). The experiment was performed twice in duplicate. *, significant difference compared to wild-type (p<0.002).

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**Figure 8. Binding is necessary for iron acquisition from ferritin.** *C. albicans* wild-type (CAF2-1), Δals3 and Δftr1 were grown on media containing ferritin as the sole source of iron. SD agar was buffered using 100 mM HEPES (pH 7.4). BPS, iron chelator; ferritin, 2 μg/ml ferritin. Cells were spotted at two concentrations (left to right, 10⁵ and 10⁶ cells, respectively) for each strain. All plates were incubated for 3 days at 37°C under 5% CO₂. The assay was performed three times.

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medium during growth even on buffered ferritin plates (Figure S2). Additionally, the fungus was only able to use ferritin as an iron source under conditions which allowed acid production (glucose, but not casamino acids as a carbon source) and acidification of the surrounding environment (low concentrations of buffer at pH 7.4). Similarly, it has been observed that the bacterial pathogen Staphylococcus aureus, under iron starvation, decreases the local pH resulting in the release of iron from transferrin [91].

It is also possible that C. albicans can produce and secrete reductants, which are able to sequester iron from ferritin. Such a process would indeed be favoured by acidification of the surrounding media. In agreement with this model, reductants or chelators such as thioglycolic acid, ascorbate, and acetol- and benzohydroxamic acids are capable of releasing iron from the ferritin core [92–94]. Underscoring the importance of pH in the release of iron from ferritin, this process is increased at pH 5.2 in comparison to pH 7.4 [94]. However, since we demonstrated that binding is necessary for ferritin iron exploitation by C. albicans, it can be hypothesized that a surface factor rather than a secreted factor is necessary for ferric iron reduction from the ferritin core. Another possible speculation is that reductases on the C. albicans cell surface can reduce ferric iron from the ferritin core and that this process may be facilitate under acidic pH.

Although we do not have experimental evidence that local acidification occurs in vivo during infection, transcriptional profiling of C. albicans during experimental infections suggests that the local environment of at least some cells in fact changes from neutral to acidic pH during invasion and tissue damage. For example, we have found that the acid induced gene, HHR2 is up-regulated during tissue invasion [34].

In addition to the ability to acidify the environment, C. albicans requires the reductive high-affinity iron uptake pathway to exploit iron from ferritin. Mutants lacking either the high-affinity permease Ftr1 or the copper transporter Ccc2 (which is essential for the reductive pathway) [63,64] did not grow on ferritin plates even when the initial pH was low. Therefore, we conclude that a combination of active acidification and uptake via the high-affinity permease are key mechanisms in this process. As a third prerequisite, we hypothesized that a close association between C. albicans cells and ferritin is required for the release of iron from ferritin and subsequent uptake into the fungal cell. This close contact is facilitated by binding of ferritin on the fungal surface.

In principle it may also be postulated that a yet unknown molecule is secreted by C. albicans, which binds ferritin and subsequently delivers the iron protein to a surface receptor, similar to some bacteria which can secrete haemophores that bind extracellular haemoglobin and mediate its delivery to surface receptors [95]. However, such a mechanism is unlikely to be involved in ferritin-binding by C. albicans since fungal cells that were killed with thimerosal and then washed, removing any secreted factors, were still able to bind ferritin.

Interestingly, fungal cells killed via exposure to UV-light lost their ability to bind ferritin. This result suggests that ferritin-binding at the cell surface is mediated by a receptor which is inactivated by UV treatment. In support of this possibility, it is known that certain proteins can be inactivated by exposure to UV light [96].

Several lines of evidence suggest that the cell surface protein, Als3 is a receptor that binds ferritin and facilitates iron acquisition from this protein. (1) Only hyphae, but not yeast cells bound ferritin and Als3 is known to be a hyphal-specific protein. However, the binding of ferritin did not need the hyphal morphology, since a mutant lacking Hgc1 [74] did not produce true hyphae, but still bound ferritin (Figures 4A and 4B) and expressed ALS3 (not shown). (2) Mutants lacking transcription factors known to regulate ALS3 expression (Tec1, Bcr1) [44,45] had a reduced ability to bind ferritin. In agreement with this, a mutant that was unable to form hyphae (Are81) and that did not express ALS3, also displayed reduced binding of ferritin and reduced growth on ferritin plates. (3) A mutant lacking ALS3 was dramatically reduced in its ability to bind ferritin and displayed poor growth on ferritin plates. The Als3+::ALS3 re-integrant strain had a restored ability to bind ferritin and a partially restored ability to grow on ferritin plates, although not to wild-type levels, possibly due to a gene dosage effect. Finally, (4) a S. cerevisiae strain expressing Als3 was able to bind ferritin.

Binding of ferritin to hyphal surfaces was observed with both exogenously added purified ferritin and during the interaction of C. albicans with intact epithelial cells. Only hyphae, but not yeast cells showed bound ferritin during interaction with epithelial cells. Furthermore, ferritin accumulation was predominantly observed on those hyphae that had invaded the epithelial cells. Finally, the hyphae of the Als3 mutant did not show ferritin accumulation.

Taken together, these data suggest that ferritin can be used as an iron source by C. albicans via direct binding by Als3 on the surface of hyphae, iron release is then mediated by acidification and uptake is facilitated by the reductive pathway (Figure 12).

Although we do not have direct evidence that ferritin is in fact used as an iron source during interaction with epithelial cells, these data at least suggest that ferritin is in close contact to invading C.

Figure 9. Als3 is a ferritin receptor. S. cerevisiae cells overexpressing ALS1, ALS3, ALSS5 (driven by the ADH promoter) or carrying an empty plasmid (pADH) were incubated for 15 min in the presence of 25 μg/ml ferritin coupled to a fluorescent dye. Cells were washed to remove non-bound ferritin and analyzed with fluorescence microscopy in duplicate repeated three times. Bar indicates 10 μm.

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albicans hyphae and thus may be exploited by the above proposed mechanism. This view is supported by the fact that both the Δals3 mutant and Δals3+ALS3 reintegrant cells were co-incubated with ferritin-enriched oral epithelial cells and differentially stained. (A), (E), (I) and (M); staining of extracellular (non-invaded) C. albicans with concanavalin A conjugated with fluorescein before cell permeabilization. (B), (F), (J) and (N); calcofluor white staining of whole C. albicans cells following epithelial cell permeabilization. (C), (G), (K) and (O); fluorescent dye (DY649) coupled antibody staining of ferritin. White arrows indicate hyphae surrounded by epithelial ferritin. (D), (H), (L) and (P); merged images. Bar in (P) indicates 10 μm.

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Figure 10. C. albicans hyphae invading oral epithelial cells bind ferritin. C. albicans wild-type (SC5314), Δals3 mutant and Δals3+ALS3 reintegrant cells were co-incubated with ferritin-enriched oral epithelial cells and differentially stained. (A), (E), (I) and (M); staining of extracellular (non-invaded) C. albicans with concanavalin A conjugated with fluorescein before cell permeabilization. (B), (F), (J) and (N); calcofluor white staining of whole C. albicans cells following epithelial cell permeabilization. (C), (G), (K) and (O); fluorescent dye (DY649) coupled antibody staining of ferritin. White arrows indicate hyphae surrounded by epithelial ferritin. (D), (H), (L) and (P); merged images. Bar in (P) indicates 10 μm.

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C. albicans Exploits Iron from Ferritin via Als3

Several studies have shown that pathogenic microbes link the availability of iron with virulence attributes. In this study, we show that a similar link between the regulation of an iron acquisition system and virulence attributes exists in C. albicans. In fact, the regulation of the ferritin receptor Als3 is independent from external iron sources and seems to be strictly linked to hyphal formation, one of the most extensively investigated virulence attributes of C. albicans [30,98].

Therefore, iron acquisition of the intracellular iron storage protein ferritin is hyphal regulated. Hyphal formation is also associated with adhesion, proteolytic activity, cellular invasion and damage [32–34,89,99], and the hyphal form of the organism is the predominant morphology that reaches the intracellular compartments of epithelial cells where ferritin is located. Therefore, C. albicans co-regulates morphology, invasion, tissue damage and an iron acquisition system. This view may explain why iron acquisition from ferritin is a hyphal-specific property and does not occur with the normally non-invasive yeast cells.
A second potential link exists between the external pH, hyphal formation and iron acquisition. It is well known that the external pH influences hyphal formation [100,101] and we recently reported that pH-dependent hyphal formation is crucial for liver invasion [34]. During liver invasion C. albicans cells are exposed to a neutral or alkaline pH and iron limited conditions as reflected by transcriptional profiles [34]. Availability of iron for fungal cells within a human host is even more difficult in neutral or slight alkaline pH conditions such as those found in the liver tissue (pH 7.4) because the balance between the soluble Fe$^{3+}$ ion and the insoluble ferric form Fe$^{3+}$ shifts towards the insoluble form [41]. Therefore, the formation of hyphae and expression of Als3 in response to neutral pH may facilitate iron acquisition by C. albicans.

Interestingly, the expression of ALS3 is not absolutely linked to the hyphal morphology in wild-type cells. Sosinska et al. [102] recently observed that hypoxic conditions and iron restriction in a vagina-simulative medium affected cell morphology and the cell wall proteome of C. albicans. One of the proteins found in yeast cells under these iron limited conditions was Als3, which indicates that even proteins which are strictly hyphal-associated under most growth conditions, may be expressed in the yeast form. Similarly, White and co-workers recently showed that C. albicans expresses a number of hyphal-specific genes (such as ECE1) in a murine gut model of commensalism, whilst growing in the yeast morphology [103]. The observation that yeast cells express Als3 under iron limited conditions may further support the view that this protein is involved in iron acquisition from the host. However, the expression of ALS3 is not directly linked to low iron conditions since two studies that analyzed the influence of iron on the genome wide gene expression of C. albicans [47,83] found that iron starvation did not increase the expression of ALS3.

The Als protein family of C. albicans encodes large cell-surface GPI-glycoproteins that were originally implicated in the process of adhesion to host surfaces [75,104]. Expression of Als3, was shown to be hyphal-specific [36] and was observed in vivo during oral and systemic infection [33,34]. In addition to its adhesion properties, Als3 was recently shown to be an invasin that binds to cadherins and induces endocytosis by host cells [32]. In this study, we made the intriguing observation that Als3 has a third function in iron acquisition by binding to host ferritin, indicating that this single member of a protein family has multiple virulence-associated functions.

Materials and Methods

Fungal growth conditions and strains
C. albicans were grown in liquid YPD medium (1% yeast extract [Merck, http://www.merck.de], 2% bactopeptone [Difco, http://www.bdbiosciences.com], and 2% D-glucose [Roth, http://www.carl-roth.de]) in a shaking incubator at 30°C for 8 h. Subsequently, the cultures were diluted 1:1000 in LIM0 medium [105] and incubated in a shaking incubator at 30°C overnight for iron starvation. For non-starved cells, precultures were incubated in YPD medium overnight at 30°C with shaking. The yeast cells were harvested by centrifugation, washed three times in filter sterilized ultra-pure water and counted using a hemacytometer. Strains of C. albicans and S. cerevisiae used in this study are listed in Table 3 and Table 4, respectively.

Oral epithelial cells
The epithelial cell line TR146, derived from a squamous cell carcinoma of buccal mucosa [106], was kindly provided by Cancer Research Technology (http://www.cancertechnology.co.uk). TR146 cells were routinely grown in RPMI 1640 medium (PAA, http://www.paa.com) supplemented with 10% fetal bovine serum in triplicate. * Significant difference compared to the wild-type (p<0.0001).

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incubator at 37°C for 8 h. Supernatants were removed for LDH measurements. All incubations were performed in a humidified incubator. After 24 h incubation, monolayers were washed twice with phosphate-buffered saline without calcium or magnesium (PBS) and serum-free RPMI 1640 medium was added, followed by incubation for 3 h. Supernatants were removed and replaced with diluted 1:1000 in PBS with 1% bovine serum albumin (BSA, Invitrogen). The same exposure time and light intensity were used to analyze all samples, permitting comparisons. For every sample, 10 random chosen fields per cover-slip were photographed using a DFC 350 FX camera (Leica). A representative picture of each condition was selected.

To monitor the ferritin content of cells, the uninfected epithelial cells were used. Using immunofluorescence, fixed monolayers were permeabilized through incubation with 0.1% Triton X-100 (Serva, http://www.serva.de) for 15 min at room temperature and washed three times with PBS. Next, the samples were blocked using Image-iT™ FX signal enhancer (Invitrogen, http://probes.invitrogen.com/products/) for 30 min at room temperature in a humidity chamber. Cells were again washed three times with PBS and incubated with rabbit anti-ferritin antibody (Sigma-Aldrich) coupled with dye DY-649 (Dyomics, http://www.dyomics.com) diluted 1:1000 in PBS with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at room temperature. Finally, cover-slips were washed three times with PBS, inverted and mounted on a microscope slide with ProLong® Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen).

### Table 3. C. albicans strains used in this study.

| Strain | Genotype                  | Reference |
|--------|---------------------------|-----------|
| SC5314 | wild-type                 | [110]     |
| CAI-4+Clp10 | ura3::imm434/ura3::imm434+Clp10 (URA3) | [111]     |
| Jals3 | ura3::imm434::URA3-RO1/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG als3::ARG4/als3::HIS1 | [76]     |
| Jals3+ALS3 | ura3::imm434::URA3-RO1/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/als3::ARG4/als3::HIS1 | [76]     |
| Tbc1 | ura3::imm434::ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG p:His1 bcr1::ARG4/bcr1::URA3 | [44]     |
| Jcc1-2 | ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/als3::ARG4/als3::HIS1 | [63]     |
| Jcc1+2::CCC2 | ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/als3::ARG4/als3::HIS1 | [63]     |
| Jche1 | ura3::imm434::URA3-RO1/ura3::imm434 ece1::hisG/ece1::hisG | [40]     |
| Jfrt1 | ura3::imm434::ura3::imm434 frt1::hisG/frt1::hisG-URA3::hisG | [64]     |
| Jfrt1+1::FRT1 | ura3::imm434::ura3::imm434 frt1::hisG/frt1::URA3-3::FRT1 | [64]     |
| Jgmc1 | ura3::imm434::ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG hgc1::ARG4/hgc1::HIS1 | [74]     |
| Jgmc1+Clp10 | ura3::imm434::ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/als3::ARG4/als3::HIS1+Clp10 (URA3) | This study |
| Jhr1 | ura3::imm434::ura3::imm434 frt1::hisG/hr1::hisG-URA3::hisG | [39]     |
| Jhr1+1 | ura3::imm434::ura3::imm434 frt1::hisG/hr1::URA3-3::FRT1 | This study |
| Jhr1+1::Clp10 | ura3::imm434::ura3::imm434 frt1::hisG/hr1::URA3-3::FRT1 | This study |
| Jhrs1 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::php | [72]     |
| Jhrs1+1::Clp10 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::php+Clp10 (URA3) | This study |
| Jhrs5 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::php | [72]     |
| Jhrs5+1::Clp10 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::php+Clp10 (URA3) | This study |
| Jhrs5+1::FRT1 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::URA3-3::FRT1 | This study |
| Jhrs5+1::FRT1+1::FRT1 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::URA3-3::FRT1 | This study |
| Jhrs5+1::FRT1+1::FRT1+1::FRT1 | ura3::imm434::ura3::imm434 ral1::hisG/ral1::URA3-3::FRT1 | This study |

### Table 4. S. cerevisiae strains used in this study.

| Strain | Genotype                  | Reference |
|--------|---------------------------|-----------|
| ATCC9763 | wild-type                 | American Type Culture Collection |
| pADH1 | leu2 his3 trp1 ura3::pADH1 | [77]     |
| pALS1 | leu2 his3 trp1 ura3::pALS1 | [77]     |
| pALS3 | leu2 his3 trp1 ura3::pALS3 | [77]     |
| pALS5 | leu2 his3 trp1 ura3::pALS5 | [77]     |

To measure epithelial cell damage, the following calculation was used: 100×(ECa−C1−C2)/100L−C1 = relative cytotoxicity

Using immunofluorescence. Briefly, fixed monolayers were permeabilized through incubation with 0.1% Triton X-100 (Serva, http://www.serva.de) for 15 min at room temperature and washed three times with PBS. Next, the samples were blocked using Image-iT™ FX signal enhancer (Invitrogen). The same exposure time and light intensity were used to analyze all samples, permitting comparisons. For every sample, 10 randomly chosen fields per cover-slip were photographed using a DFC 350 FX camera (Leica). A representative picture of each condition was selected.

### Epithelial cell monolayer damage assay

Epithelial cell damage caused by C. albicans, was determined by the release of lactate dehydrogenase (LDH) into the medium using a Cytotoxicity Detection Kit–LDH (Roche, http://www.roche.de). The assays were performed according to the manufacturer instructions and the measurements were performed in duplicates.

To measure epithelial cell damage, the following calculation was used: 100×(ECa−C1−C2)/100L−C1 = relative cytotoxicity

Additional conditions: (1) RPMI 1640 with 50 μM bathophenanthroline disulfonate acid disodium salt (BPS; iron chelator; Sigma-Aldrich, http://www.sigmaaldrich.com); (2) RPMI 1640 with 10% FBS; (3) RPMI 1640 with 10% FBS and indicated concentrations of iron chloride (FeCl3; Merck). After 24 h incubation, monolayers were additionally incubated for 24 h in three different conditions: (1) RPMI 1640 with 50 μM bathophenanthroline disulfonate acid disodium salt (BPS; iron chelator; Sigma-Aldrich, http://www.sigmaaldrich.com); (2) RPMI 1640 with 10% FBS; (3) RPMI 1640 with 10% FBS and indicated concentrations of iron chloride (FeCl3; Merck). After 24 h incubation, monolayers were washed twice with phosphate-buffered saline without calcium or magnesium (PBS) and serum-free RPMI 1640 medium was added. Monolayers were washed twice with phosphate-buffered saline without calcium or magnesium (PBS) and serum-free RPMI 1640 medium was added.
Ferritin agar plates

To investigate whether C. albicans was able to grow with ferritin as the sole source of iron, we added 350 \( \mu \)M BPS to the SD agar medium (6.7 g/l yeast nitrogen base, YNB [Difco]; 20 g/l D-glucose; 20 g/l purified agar [Oxoid, http://www.oxoid.com]). Additionally, HEPES buffer (Sigma-Aldrich) was added to the medium as indicated and the pH was adjusted to 7.4 using a 5 M NaOH stock solution (Roth). To prevent active acidification of the medium by the fungus, 20 g/l casamino acids (Difco) was used in place of D-glucose. The ferritin solution (ferritin from horse spleen [Sigma-Aldrich]) was diluted 1:100 in a dilution buffer (5 mM HEPES; 0.1 M NaCl [Roth]) and passed through a Microcon YM-10 Centrifugal Filter Unit (Millipore, http://www.millipore.com). The retentate was collected in a fresh 1.5 ml microcentrifuge-tube and the original volume was adjusted with the dilution buffer. Afterwards, this ferritin solution was plated out on agar surfaces at indicated concentrations. To monitor the pH changes in the medium during C. albicans growth, the pH indicator bromocresol green (Sigma-Aldrich) was added to the medium at a concentration of 3.9 mg/l.

Ferritin binding assay

C. albicans cells growing under iron limitation, as described above, were washed and enumerated. Approximately 5 \( \times \) 10^5 cells were added per well in a 24 well plate (TPP, http://www.tissue-cell-culture.com) containing Poly-L-Lysine-coated (Biorchim AG, http://www.biorchim.de) 12-mm diameter glass cover-slips and 1 ml RPMI 1640. The cells were incubated for 3 h at 37°C under 5% CO2 to induce hyphae. Afterwards, the cells were washed once with PBS and incubated for 1 h in 1 ml PBS with 1% bovine serum albumin (BSA) and 100 \( \mu \)g/ml ferritin. Subsequently, the cells were washed three times with PBS to remove non-bound ferritin and fixed with 500 \( \mu \)l Roti®-Histofix 4%.

To test if viability is necessary for ferritin binding, C. albicans hyphae (3 h in RPMI 1640 at 37°C and 5% CO2) were killed using two different approaches: either 1.5 h incubation at room temperature with 0.05% Thimerosal (Sigma-Aldrich) or 2 times exposition to 0.5 J/cm² UV light in a UV-crossliner with a 254 nm low pressure mercury-vapor lamp (Vilber-Loumart, http://www.vilber.de). Complete killing without residual viability of cells was checked by plating the cells on YPD agar plates. After killing, the cells were incubated with ferritin and fixed as described above.

The fixed cells were washed three times with PBS and incubated with rabbit anti-ferritin antibody coupled with dye DY-649 diluted 1:500 in PBS with 1% BSA for 1 h at room temperature. Next, the cover-slips were inverted and mounted on a microscope slide with ProLong® Gold Antifade Reagent (Invitrogen) and cells were visualized using a Leica DM 5500B microscope (Leica). Photomicrographs were taken using a DFC 350 FX camera (Leica). To quantify how many C. albicans cells bound ferritin, at least 100 cells per cover-slip were counted and percent binding was calculated by counting the total number of cells and the number of cells displaying fluorescent signal. All binding assays were performed in duplicates. Cells incubated without ferritin were used as a negative control.

Because S. cerevisiae cells were detached during the washing steps described above, a different approach was used. The use of a fluorophore-coupled ferritin reduced the number of washing steps in the staining procedure and consequently left more cells on the coverslip for observation by fluorescent microscopy. Briefly, 5 \( \times \) 10^5 cells were added per well in a 24 well plate containing Poly-L-Lysine-coated 12-mm diameter glass cover-slips in 1 ml RPMI 1640. The cells were incubated for 1 h at 30°C. Afterwards, the medium was removed and 250 \( \mu \)l PBS with 1% BSA and 25 \( \mu \)g/ml ferritin coupled with dye DY-649 was added. After 15 min at 30°C, the cells were washed once with PBS, fixed, mounted and visualized under the microscope as described above for C. albicans cells.

Transmission electron microscopy

C. albicans wild-type cells (SC5314) were grown on poly-L-lysine-coated cover-slips (0.3 mm in diameter) in the presence or absence of 100 \( \mu \)g/ml ferritin for 6 h in RPMI 1640. Afterwards, the cells were washed with PBS four times to remove non-bound ferritin and then immersed in fixative (4% formaldehyde, prepared from para-formaldehyde [Roth] and 0.1% glutaraldehyde [Roth] in 0.05 M HEPES ) at room temperature. After three min the fixative was replaced with fresh fixative and stored at 4°C overnight. The samples were dehydrated in ethanol (Roth) by progressively lowering the temperature to 5°C and infiltrated with Lowicryl K4M resin (Polysciences, http://www.polysciences.com) at 4°C (107). The resin polymerization was carried out under UV light at −35°C for 24 h and for 10 h at 0°C. Ultra thin sections (60–80 nm thick) were produced with an Ultracut S (Leica) and a diamond knife. Sections were collected on formvar coated copper slot grids. Bright-field transmission electron microscopy was performed with an EM902 (ZEISS, http://www.zeiss.de) at 80 kV. Images were recorded with a 1 k CCD camera (Proscan, http://www.proscan.de).

Flow cytometry analysis of ferritin binding

Flow cytometry was used to quantify the binding of ferritin on the surface of C. albicans hyphal cells. C. albicans cells were grown under iron limitation, as described above, washed and counted. Approximately 10^6 cells in 1 ml RPMI 1640 medium were added to poly-L-lysine-coated (Biorchim) 12-mm diameter glass cover-slips in a 24 well tissue-culture plate (TPP). The cells were incubated for 2 h at 37°C in 5% CO2 to induce hyphae. Next, the cells were washed once with PBS and incubated for 1 h in 0.5 ml PBS with 1% bovine serum albumin (BSA) and 100 \( \mu \)g/ml ferritin. Subsequently, the cells were washed three times with PBS to remove non-bound ferritin and fixed with 500 \( \mu \)l Roti®-Histofix 4%.

To test if viability is necessary for ferritin binding, C. albicans hyphae (3 h in RPMI 1640 at 37°C and 5% CO2) were killed using two different approaches: either 1.5 h incubation at room temperature with 0.05% Thimerosal (Sigma-Aldrich) or 2 times exposition to 0.5 J/cm² UV light in a UV-crossliner with a 254 nm low pressure mercury-vapor lamp (Vilber-Loumart, http://www.vilber.de). Complete killing without residual viability of cells was checked by plating the cells on YPD agar plates. After killing, the cells were incubated with ferritin and fixed as described above.

The fixed cells were washed three times with PBS and incubated with rabbit anti-ferritin antibody coupled with dye DY-649 diluted 1:2000 in PBS with 1% BSA for 1 h at room temperature. Next, the cover-slips were inverted and mounted on a microscope slide with ProLong® Gold Antifade Reagent (Invitrogen) and cells were visualized using a Leica DM 5500B microscope (Leica). Photomicrographs were taken using a DFC 350 FX camera (Leica). To quantify how many C. albicans cells bound ferritin, at least 100 cells per cover-slip were counted and percent binding was calculated by counting the total number of cells and the number of cells displaying fluorescent signal. All binding assays were performed in duplicates. Cells incubated without ferritin were used as a negative control.

Because S. cerevisiae cells were detached during the washing steps described above, a different approach was used. The use of a fluorophore-coupled ferritin reduced the number of washing steps
Concanavalin A–fluorescein conjugate (Invitrogen) in PBS for 45 min at room temperature. After washing, the cells were permeabilized by incubation with 0.1% Triton X-100 for 15 min at room temperature. After washing three times with PBS, the samples were blocked using Image-iT™ FX signal enhancer (Invitrogen) for 30 min at room temperature in a humidity chamber. After washing three times with PBS, the cells were incubated with rabbit anti-ferritin antibody coupled with dye DY-649 diluted 1:1000 in PBS with 1% BSA for 1 h at room temperature. To stain C. albicans cells localized outside and inside epithelial cells, the samples were incubated with 10 μg/ml Calcofluor White (Sigma) in 0.1 M Tris-hydrochloride (pH 9.0 [Roth]) for 20 min at room temperature.

Finally, cover-slips were washed three times with ultra pure water, inverted and mounted on a microscope slide with ProLong® Gold Antifade Reagent. At least two experiments in duplicates were analyzed using a Leica microscope and 10 randomly chosen fields per cover-slip were photographed. A representative picture of each strain was selected.

**Invasion of ferritin depleted or enriched oral epithelial cells**

Ferritin depleted or enriched oral epithelial cell monolayers (as described above) were washed twice with PBS and infected with ~10^5 iron starved C. albicans cells in serum-free RPMI 1640 medium for 3 h. The samples were washed twice with PBS and fixed with 500 μl Roti®-Histofix 4%. C. albicans cells alone were incubated separately and used as control. All samples were incubated in a humidified incubator at 37 °C and 5% CO₂. The samples were stained to distinguish invading from non-invading fungal cells as described above. At least 100 randomly selected organisms were analyzed and the percentage of organisms that had invaded the epithelial cells was calculated.

**Sample preparation for RNA extraction**

C. albicans cells growing under iron limitation, as described above, were washed and enumerated. Approximately 2 × 10^6 cells were added per well in a 24 well plate containing Poly-L-Lysine - coated 12-mm diameter glass cover-slips in 1 ml RPMI 1640 with 100 μg/ml ferritin. The strains used were CA14 carrying Clp10; Δatg1 carrying Clp10 and Δars1 carrying Clp10. The plasmid Clp10 was used to reconstitute CRA4 into the RP10 locus of each strain [108]. After 1.5 h incubation at 37 °C under 5% CO₂, the medium was removed and 100 μl peqGOLD RNAPure (PeqLab, http://www.peqlab.de) was added per well. The cells were immediately removed from the cover-slips using a pipette point. For each strain, cells from 12 wells were pooled in a microcentrifuge tube and immediately shock frozen in liquid nitrogen. To verify that ferritin was bound to C. albicans hyphae as observed before, additional cover-slips for each strain were fixed and ferritin was stained as described.

**RNA extraction and labeling.** Frozen cells were lysed and homogenized (Precellys 24, PeqLab) with glass beads (0.5 mm, Roth). Total RNA was extracted as previously described [109]. Total RNA was linearly amplified and labeled using the ‘Low RNA Input Fluorescent Linear amplification Kit’ (Agilent Technologies, http://www.agilent.de).

**Microarray hybridization and analysis**

For transcriptional profiling, C. albicans microarrays (Eurogentec) were used as previously described [109]. RNA was co-hybridized with a common reference (RNA from SC5314 grown in YPD medium, mid-log phase, 37 °C). Slides were hybridized, washed and scanned as described [109]. Data normalization (LOWESS) and analysis were performed in GeneSpring 7.2 software (Agilent Technologies). Reliable expression of genes was defined as normalized expression of present genes that did not vary more than 1.5 standard deviations within replicate arrays. Genes were defined as differentially expressed if their expression was at least 2 times stronger or 2 times weaker in at least one strain compared to the common reference. Using the Benjamini and Hochberg false discovery test, a p-value < 0.05 was considered as significant. Microarray data from four independent experiments (two of them with dye swap) were used. To identify genes involved in ferritin binding, genes were selected that were up-regulated (≥2.5 increase in expression compared to the common reference) in wild-type and Δatg1 cells, but unaltered or down-regulated (≤1.5 of the common reference expression) in the Δars1 mutant. Raw data have been deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE11490.

**Statistical Analysis**

Statistical significances (p-values) were calculated with the Student’s two-tailed t-test function in Microsoft Excel, with exception of the microarray analysis described above.

**Supporting Information**

**Figure S1**  C. albicans can acidify the medium during growth on ferritin plates. C. albicans wild-type (SC5314) was grown on media containing ferritin as the sole source of iron and bromocresol green (3.9 mg/ml) as a pH indicator. SD agar was buffered using 25 mM HEPES (pH 7.4). BPS, iron chelator; ferritin, 15 μg/ml ferritin. All plates were incubated for 4 days at 37 °C under 5% CO₂. Blue indicates pH values higher than 5.5. Green indicates pH values between 5.5 and 4. Yellow indicates pH values below 4. The assay was performed twice in duplicate.

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**Figure S2**  Examples of ferritin plates as described in Table 1. SD agar was buffered using 100 mM HEPES (pH 7.4). BPS, iron chelator. Ferritin, 5 μg/ml ferritin. All plates were incubated for 3 days at 37 °C under 5% CO₂.

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**Figure S3**  Ferritin binding does not require live cells or iron limitation and is UV sensitive. (A) Comparison of ferritin binding between live and dead cells (using thimerosal or UV light). (B) Cells from iron limitation medium (LIM0) or from YPD were used for the ferritin binding assay. Additionally, cells from the same YPD preculture were tested for ferritin binding with the addition of 50 μM iron chloride during the binding assay.

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**Figure S4**  Growth of selected mutants on ferritin plates. SD agar was buffered using 100 mM HEPES (pH 7.4). BPS, iron chelator. Ferritin, 5 μg/ml ferritin. All plates were incubated for 3 days at 37 °C under 5% CO₂.

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**Figure S5**  Invasion of epithelial cells by Δatg1. Approximately 10^5 iron starved wild-type C. albicans cells (SC5314) or Δatg1 mutant cells were co-incubated with epithelial cells for 3 h. After fixation the samples were differentially stained and analysed under the fluorescence microscope. The experiment was performed three times in duplicate. No significant difference was observed between the wild-type strain and the Δatg1 mutant strain.

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Author Contributions

Conceived and designed the experiments: RSA SB AA JEEJ SGF BH. Performed the experiments: RSA ML. Analyzed the data: RSA BH. Wrote the paper: RSA SGF BH.

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