Host adaptation, avirulence and antivirulence genes of *Candida glabrata*

Dissertation

To Fulfill the Requirements for the Degree of
"Doctor of Philosophy" (PhD)

Submitted to the Council of the Faculty of Biological Sciences
of the Friedrich Schiller University Jena

by Master of Science (M.Sc.)

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This thesis was prepared at the “Leibniz Institute for Natural Product Research and Infection Biology – Hans Knoell Institute (HKI)”, Jena in the Department of Microbial Pathogenicity Mechanisms (MPM) under the supervision of Prof. Bernhard Hube. This study was financed by the HKI, the German Federal Ministry of Education, and by the Excellence Graduate School Jena School for Microbial Communication (JSMC).

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and Infection Biology
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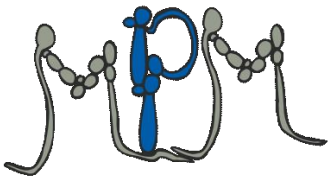

Bundesministerium
für Bildung
und Forschung

PhD Defense’s date: 24/09/2021

Reviewers:

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Caminante, son tus huellas
el camino y nada más;
Caminante, no hay camino,
se hace camino al andar.
Al andar se hace el camino,
y al volver la vista atrás
se ve la senda que nunca
se ha de volver a pisar.
Caminante, no hay camino
sino estelas en la mar.

Antonio Machado

(And you never walk alone! BH)

1. Contents

2. Summary

One billion people worldwide are affected by fungal pathogens, of which 1.6 million are killed per year – more deaths than by malaria and roughly equal to the number of deaths by tuberculosis. A further understanding on how these species adapt to the host and how this influences their pathogenicity is crucial to develop successful strategies to prevent, diagnose and treat fungal infections. Toward this aim, this study introduces the concepts of antivirulence and avirulence genes in human fungal pathogens, based on the well-established concepts of antivirulence genes in bacterial pathogens and avirulence genes in phytopathogenic fungi. Several relevant analogies are presented here, which will help us to understand better the pathogenicity of fungi that infect humans: The emergence and evolution of virulence through gene loss or inactivation of the so-called antivirulence genes; and the reduction of the pathogen's virulence due to the host recognition of avirulence factors. In a comparative approach to the evolution of fungal pathogenesis, this work also shows the co-evolution of the most common *Candida* pathogens with host epithelial cells, which led to a general pro-commensal epithelial response in absence of damage, and an immune-stimulatory response depending on the species-specific damage.

Candida glabrata is the second most frequent cause of candidiasis despite its inability to cause epithelial damage and inflammatory response *in vitro* or in mice models. The main adaptations of this fungus to infection situations are its ability to survive and replicate within the phagosome of macrophages, and its high intrinsic resistance to stress and antifungals. This work shows that *C. glabrata* seems to rely at least partially on a phenotypic variant, the *petite* phenotype, which through loss of mitochondrial function can increase its success as a pathogen. Despite showing a decrease in fitness, yeasts with the *petite* phenotype are able to resist high concentrations of azole antifungals and survive better within macrophages. Importantly, a cross-resistance effect between these two exists, since *C. glabrata* turns *petite* upon exposure to azoles and after phagocytosis, showing for the first time a close connection between antifungal resistance and immunoresponse in a pathogenic fungus of humans. This may be of clinical relevance, as the *petite* phenotype was found among clinical isolates.

In addition, this study elucidates further relevant pathways for the survival and persistence of *C. glabrata* within macrophages, especially for long-tern events after phagocytosis. One aspect is the accumulation of trehalose, which in some

microorganisms serves as protection against oxidative stress as well as an energy source after stress exposure. Here, deletion mutants of the putative trehalase enzymes of *C. glabrata* show a decrease in virulence and ability to survive phagocytosis after four days, thus uncovering a new important pathway involved in adaptation of *C. glabrata* to the host. Furthermore, the ability of this fungus to replicate within macrophages has been attributed to the utilization of alternative carbon sources in the phagosome, although the use of certain carbon sources, like acetate, seems to be detrimental for the survival of *C. glabrata* within macrophages. This work shows how the absence of the antivirulence genes encoding acetate transporters benefits long-term survival of *C. glabrata* within macrophages. Together, these results shed light on the metabolic pathways involved in replication and persistence of *C. glabrata* within these immune cells.

In summary, this work introduces new concepts for a better understanding of the emergence and evolution of pathogenic fungi of humans, and focuses on elucidating so-far unknown strategies of the opportunistic pathogen *C. glabrata*: the loss of mitochondrial function can be beneficial for this fungus under infection, and the metabolism of trehalose and acetate influence its virulence potential by affecting its survival within the phagosome.

3. Zusammenfassung

Ungefähr eine Milliarde Menschen werden jährlich weltweit mit Pilzerregern infiziert, von denen 1,6 Millionen sterben. Das sind mehr Menschen als durch Malaria und etwa ebenso viele wie durch Tuberkulose ums Leben kommen. Ein besseres Verständnis darüber, wie sich diese pathogenen Pilzarten an den Wirt anpassen und wie dies ihre Pathogenität beeinflusst, ist entscheidend für die Entwicklung erfolgreicher Strategien zur Prävention, Diagnose und Behandlung von Pilzinfektionen. Zu diesem Zweck werden in dieser Studie die Konzepte der Antivirulenz- und Avirulenzgene bei humanen Pilzpathogenen vorgestellt, die auf den gut etablierten Konzepten der Antivirulenzgene bakterieller Pathogene und der Avirulenzgene phytopathogener Pilze basieren. Es werden einige relevante Analogien vorgestellt, die uns helfen werden, die Pathogenität von Pilzen, die den Menschen infizieren, besser zu verstehen: Die Entstehung und Evolution der Virulenz durch Genverlust oder Inaktivierung der sogenannten Antivirulenzgene sowie die Reduktion der Virulenz des Erregers durch die Wirtserkennung von Avirulenzfaktoren. In einem vergleichenden Ansatz zur Evolution der Pilzpathogenese zeigt diese Arbeit auch die Co-Evolution der häufigsten *Candida*-Pathogene mit Wirtsepithelzellen, welche bei fehlender Schädigung zu einer generell pro-kommensalen Epithelantwort und in Abhängigkeit von der artspezifischen Schädigung zu einer immunstimulierenden Antwort führte.

Candida glabrata ist trotz fehlender Epithelschädigung und Entzündungsreaktion *in vitro* und bei Mäusen die zweithäufigste Ursache der Candidiasis beim Menschen. Die wichtigsten Anpassungen dieses Pilzes an Infektionssituationen sind seine Fähigkeit im Phagosom von Makrophagen zu überleben und zu replizieren sowie seine hohe intrinsische Resistenz gegenüber Stress und Antimykotika. Diese Arbeit zeigt, dass *C. glabrata* zumindest teilweise auf eine phänotypische Variante, den *petite*-Phänotyp, angewiesen ist, die durch einen Verlust der mitochondrialen Funktion den Erfolg als Pathogen steigern kann. Hefen des *petite*-Phänotyps sind trotz ihrer geringeren Fitness in der Lage, hohen Konzentrationen von Azol-Antimykotika zu widerstehen und besser in Makrophagen zu überleben. Nennenswert ist, dass ein Kreuzresistenzeffekt zwischen diesen beiden Prozessen besteht, da *C. glabrata* bei Exposition gegenüber Azolen und nach Phagozytose *petite* wird, so dass in dieser Arbeit zum ersten Mal ein enger Zusammenhang zwischen antimykotischer Resistenz eines pathogenen Pilzes und der humanen Immunreaktion aufgezeigt wird. Dies könnte von klinischer Relevanz sein, da der *petite*-Phänotyp unter klinischen Isolaten gefunden wurde.

Darüber hinaus klärt die vorliegende Studie weitere relevante Wege für das Überleben und die Persistenz von *C. glabrata* innerhalb von Makrophagen auf, insbesondere für Langzeitereignisse nach der Phagozytose. Ein Aspekt ist die Akkumulation von Trehalose, die in einigen Mikroorganismen sowohl als Schutz vor oxidativem Stress als auch als Energiequelle nach Stressbelastung dient. Deletionsmutanten der putativen Trehalase-Enzyme von *C. glabrata* zeigen eine Abnahme der Virulenz und der Fähigkeit, die Phagozytose vier Tage lang zu überleben, und decken damit einen neuen wichtigen Weg auf, der an der Anpassung von *C. glabrata* an den Wirt beteiligt ist. Weiterhin wurde die Fähigkeit dieses Pilzes sich in Makrophagen zu vermehren auf die Nutzung alternativer Kohlenstoffquellen im Phagosom zurückgeführt, obwohl die Verwendung bestimmter Kohlenstoffquellen, wie Acetat, für das Überleben von *C. glabrata* in Makrophagen nachteilig zu sein scheint. Diese Arbeit zeigt, dass das Fehlen der Antivirulenz-Gene, die für Acetat-Transporter kodieren, das langfristige Überleben von *C. glabrata* innerhalb humaner Makrophagen begünstigt. Zusammengenommen geben diese Ergebnisse Aufschluss über die Stoffwechselwege, die an der Replikation und Persistenz von *C. glabrata* innerhalb dieser Immunzellen beteiligt sind.

Zusammenfassend stellt diese Arbeit neue Konzepte für ein besseres Verständnis der Entstehung und Evolution pathogener Pilze des Menschen vor und konzentriert sich dabei auf die Aufklärung bisher unbekannter Strategien des opportunistischen Erregers *C. glabrata*: Der Verlust der Mitochondrienfunktion kann für diesen Pilz während der Infektion von Vorteil sein. Außerdem wirkt sich der Stoffwechsel von Trehalose und Acetat auf sein Virulenzpotenzial aus, da dieser das intraphagosomale Überleben des Pilzes beeinflusst.

4. Introduction

4.1 The kingdom Fungi

The kingdom fungi is considered to be a major eukaryotic lineage, estimated to encompass 5.1 million species (Blackwell 2011) – similar in diversity to animals and comprising ten times more species than the kingdom plants. Fungi can be found everywhere, from the stratosphere, deserts, to deep ocean sediments, antarctic glaciers, and even our gut (Naranjo-Ortiz and Gabaldon 2019). Fungi have been crucial for the colonization of land by plants (Humphreys, Franks *et al*. 2010). For millions of years both organisms have co-evolved while maintaining diverse types of relationships: from symbiotic to biotrophic fungi, which feed on living plants; necrotrophic, which kill the plants to feed on them; and saprophytic, which feed on already dead plants. Fungi have also established mutualistic associations with animals. Arthropods, like ants and termites, are known to cultivate and feed on fungi (Gordon and Phillips 1998); ruminants, like cows or moose, rely on anaerobic fungi to degrade the cellulose in their rumen (Gordon and Phillips 1998). We humans are also colonized by different species of fungi, which constitute our mycobiota. The most common genera identified in the human gastrointestinal tract are *Candida, Penicillium, Wallemia, Cladosporium*, *Saccharomyces*, *Aureobasidium*, *Aspergillus*, and *Malassezia* (Ghannoum, Jurevic *et al*. 2010, Hoffmann, Dollive et al. 2013, Dupuy, David *et al*. 2014, Lewis, Chen *et al*. 2015, Limon, Skalski *et al*. 2017, Sokol, Leducq *et al*. 2017). Although the gut mycobiota represents less than 1% of the total flora in the gastrointestinal tract (Qin, Li *et al*. 2010, Arumugam, Raes *et al*. 2011), more and more studies show how it can support a healthy immune response and therefore establish and maintain not only commensal but also mutualistic relationships with the host (reviewed by Iliev and Leonardi 2017, Limon, Skalski *et al*. 2017, Lai, Tan *et al*. 2019).

However, fungi are also important contributors to human disease. Fungal-host relationships in general can be understood as depending on whether any and if so, how much damage is elicited by the interaction. Based on this measure, they can be divided into commensalism, colonization, and disease, in order from no damage to significant damage (Pirofski and Casadevall 2008). More than 500 species of fungi are able to cause disease in humans. Inflammatory bowel disease, allergic airways diseases, and dermatitis, among others, have been associated with alterations in fungal abundance and species composition, with a strong contribution of host genetic factors, age, life style, and bacterial microbiota (Lai, Tan *et al*. 2019). Life-threatening fungal infections mainly

occur as opportunistic interactions, meaning that the host is usually in a position of immunodeficiency or illness: Immunodeficiency, due to neutropenia, corticosteroids treatment, chemotherapy or HIV infection, constitutes an important risk factor for invasive opportunistic fungal infections (Badiee and Hashemizadeh 2014). Moreover, dysbiosis, a significant change in the normal microbiome as a result for example of antibiotic treatment, is known to predispose the host to superficial and invasive fungal infections (Oksala 1990, Samonis, Gikas *et al*. 1993, Abbott 1995, Ben-Ami, Olshtain-Pops *et al*. 2012).

4.2 Host-fungal co-evolution: The host side

More than 1.5 million people are killed annually by fungi, which in total affect over a billion people. Specifically, skin, nail and hair fungal infections are estimated to affect nearly a billion people, many millions suffer from mucosal candidiasis, and serious fungal diseases affect more than 150 million people (Bongomin, Gago *et al*. 2017). The life-threatening fungal infections are frequently associated with high mortality rates reaching up to 95% (Pfaller and Diekema 2007, Brown, Denning *et al*. 2012, Enoch, Yang *et al*. 2017). Fungi must have the ability to overcome many host protective mechanisms to cause infection, such as an ambient temperature of 37°C (in the human body) and the host immune response (Kohler, Hube *et al*. 2017). In addition, pathogenic fungi must be capable to attach to and invade a range of different tissues and acquire nutrients during infections (Kohler, Hube *et al*. 2017).

The high body temperature in mammals constitutes an important protection against fungal diseases, to such an extent that it likely provided a strong evolutionary survival advantage for these animals. Indeed, it has been proposed that endothermy and homeothermy have been fundamental evolutionary traits that mammals and birds developed as a potent nonspecific defense against most fungi (Robert and Casadevall 2009, Casadevall 2012). In fact insects, which are ectotherm animals, fall prey to many fungal species (Ortiz-Urquiza, Luo *et al*. 2015, Shang, Feng *et al*. 2015), and when infected, they rise their body temperature by a so-called “behavioral fever” to increase their survival chances (Ouedraogo, Goettel *et al*. 2004, Anderson, Blanford *et al*. 2013). Furthermore, the adaptive immune response of vertebrates confers them with a strong protection against mycotic infections, and the activation of this arm of the immune system is critical to eradicate the fungal infection (Verma, Wuthrich *et al*. 2014). Therefore, the potent mammalian resistance to fungi is likely due to a combination of vertebrate-level immunity and endothermy as a result of (among other factors) selection by pathogenic fungi (Casadevall 2012).

Nevertheless, all animals, from sponges to humans, also rely on a generic immune response that is able to recognize fungal pathogen-associated molecular patters (PAMPs), such as the abundant fungal cell wall component β-(1,3)-glucan, and prevent fungal invasion. In mammals this first response is taken care of by the innate immune system, which mainly uses phagocytosis by neutrophils and macrophages as antifungal mechanisms: After recognition of PAMPs, neutrophils in the blood stream or in infected tissues and tissue-resident macrophages engulf these microorganisms into vesicles called phagosomes, where production of reactive oxygen species (ROS), a strong decrease in pH, and influx of lytic enzymes will kill most phagocytosed pathogens (Drummond, Gaffen *et al*. 2014, Lionakis, Iliev *et al*. 2017). Neutrophils can also employ extracellular chromatin-based traps called Neutrophil Extracellular Traps (NETs) to capture and inhibit the growth of microorganisms when the fungal structures, such as hyphae or aggregates, are too large to phagocytose (Drummond, Gaffen *et al*. 2014, Lionakis, Iliev *et al*. 2017).

Mucosal epithelial cells are in constant interaction with commensal and pathogenic microbes and therefore also take part in the immune response towards these microorganisms. Intestinal epithelial cells, which are colonized by a diverse range of microbes, can prevent disease or promote commensalism by dampening pro-inflammatory responses through glucocorticoids and type I interferon signaling (Sato, Hata et al. 1998, Cima, Corazza *et al*. 2004, Coste, Dubuquoy *et al*. 2007, Munakata, Yamamoto *et al*. 2008, Kotredes, Thomas *et al*. 2017, Soderholm and Pedicord 2019). In the case of colonization by fungi, this has not been shown yet. Rather, studies have focused mainly on the host response when damage is the outcome of the host-fungal interaction: As invading fungi disrupt host tissues through hyphae and digestive enzymes, the host epithelial cells release antimicrobial peptides and cytokines to promote a protective immune response (Abbott 1995, Drummond, Gaffen *et al*. 2014, Lionakis, Iliev *et al*. 2017, Salazar and Brown 2018). During vaginal infection the toxin candidalysin, produced by *C. albicans*, damages the epithelial cells causing the release of endogenous molecules, called Damage-Associated Molecular Patterns (DAMPs), which activates the immune system promoting pathological inflammatory responses, also called immunopathology (Moyes, Wilson *et al*. 2016, Richardson, Willems *et al*. 2018).

As a last antifungal immune barrier, the host relies on nutrient depletion, termed “nutritional immunity”, by which essential metals such as iron, zinc or copper are sequestrated. These micronutrients are required by pathogens as co-factors for

respiration or oxidative stress detoxification (Hood and Skaar 2012).

4.3 Host-fungal co-evolution: The fungal side

In addition to host susceptibility as a major factor in the development of disease, fungal pathogens require virulence factors (Brunke, Mogavero *et al*. 2016). Conventionally, virulence factors refer to microbial attributes that cause direct host damage and are not involved in the viability of the microbe, with a classical example being toxins (Casadevall and Pirofski 1999). However, other microbial attributes can trigger, for example, inflammatory self-damaging host responses and therefore can also be considered (indirectly acting) virulence factors. Similarly, the damage exerted by conventional virulence factors can indirectly lead to a stronger host inflammatory response that then increase the damage outcome (Casadevall and Pirofski 2001, Pirofski and Casadevall 2008). Therefore, we have (as part of this work) suggested a new, extended concept, where a virulence factor refers to all those microbial attributes that directly or indirectly lead to host damage (Siscar-Lewin, Hube *et al*. 2019). Therefore, a virulence factor is defined in the context of a host and not by an intrinsic quality alone.
In human fungal pathogens, such virulence factors can be involved in surpassing the host barriers (temperature and immune response), in attachment, invasion, and nutrient acquisition. Similar attributes and functions can often be found in both commensal and environmental opportunistic fungal pathogens, but are generally considered to have emerged from different selection processes (Marcos, de Oliveira *et al*. 2016, Kohler, Hube *et al*. 2017).

Kaemmer *et al*. and Pekmezovic *et al*. have shown that different opportunistic *Candida* species exhibit species-specific transcriptional responses and patterns of pathogenicity when incubated with human blood or with epithelial cells, respectively (Kaemmer, McNamara *et al*. 2020; Pekmezovic, Hovhannisyan *et al*. 2021). This agrees with the hypothesis that pathogenesis has emerged several times in commensal fungi that are also opportunistic pathogens, and the pressures that lead to virulence or to an increase in virulence are not yet well understood. For instance, the phylogenetically closely related species, *C. albicans* and *C. dubliniensis*, show different adaptations to the host and differ in their virulence potential, although they both are considered human commensals and opportunistic pathogens (Moran, Coleman *et al*. 2012). Moreover, environmental opportunistic pathogens, like *Aspergillus*, *Cryptococcus* or *Coccidioides* spp. exhibit sets of efficient host adaptations and immune evasion strategies that trigger severe and persistent infections, despite the human host not being their primary niche (Moran, Coleman *et al*. 2011, Kohler, Hube *et al*. 2017). In addition, virulence factors differ

drastically even between species with a high prevalence and incidence in the clinical settings, like the damaging species *C. albicans* and *C. glabrata*, where the latter does not form hyphae nor elicit significant damage or immune responses. Yet, both are considered important human commensals and opportunistic pathogens (Brunke and Hube 2013, Enoch, Yang *et al*. 2017). These examples point out the complexity and variety in the emergence and evolution of virulence in fungi.

By broadening our view of the field of pathogenesis to include bacterial pathogens and plant-pathogenic fungi, we can better understand the emergence of virulence and the specificities of virulence factors in fungal pathogens of humans. On the one hand, in the field of bacterial pathogens the term antivirulence genes is commonly used to describe those genes that encode factors detrimental for the virulence phenotype, and which tend to be lost as the microorganisms specialize more and more in a pathogenic lifestyle (Bliven and Maurelli 2012). On the other hand, in the field of plant-pathogenic fungi, the concept of avirulence genes is widely used, which refers to those genes encoding virulence factors that, as a result of host-pathogen co-evolution, are specifically recognized by the host immune system and trigger a host's protective response against the pathogen (Lo Presti, Lanver *et al*. 2015). Evolutionary speaking, analogous processes should be at work in the human-fungal interactions, and this study therefore includes a closer look at concepts in the light of fungal pathogenesis in humans (Siscar-Lewin, Hube *et al*. 2019).

4.3.1 Commensal fungi

The commensal yeast *C. albicans* has been associated with humans since the early hominid evolution (Lott, Fundyga *et al*. 2005), and it resides in the oral cavity, vagina, and gastrointestinal tract of healthy humans as a harmless commensal. However, during this long and close relationship with its host it seems that *C. albicans* has gained or improved its ability to surpass the protective host barriers, likely via the so-called "commensal virulence school" (Hube 2009). Today, this species is the main etiologic agent responsible for mucosal disease and nosocomial invasive candidiasis globally, with a mortality rate of approximately 40% (Guinea 2014, Bongomin, Gago *et al*. 2017). The main virulence trait of *C. albicans* is its ability to filament by forming hyphae. In this morphology, *C. albicans* is able to cross the epithelial barriers and invade the host tissues and cause cell damage by secretion of the toxin candidalysin. This peptide is one of the few "classical virulence factor" identified in human pathogenic fungi so far (Moyes, Wilson *et al*. 2016, Allert, Forster *et al*. 2018, Naglik, Gaffen *et al*. 2019). Interestingly, one of the strongest filament-inducing conditions is a combination of body temperature

and serum, pointing out the adaptation of the pathogenicity mechanisms of *C. albicans* to the conditions in the human host (Ernst 2000, Thompson, Carlisle *et al*. 2011). Additional hyphae-associated genes, such as genes encoding adhesins and proteinases are uniquely expressed under filamentation and are involved in attachment to host cells and nutrient acquisition, respectively (Hube, Sanglard *et al*. 1997, Theiss, Ishdorj *et al*. 2006, Naseem, Araya *et al*. 2015). Furthermore, *C. albicans* also uses its hyphae to escape from phagocytes as it can filament within the phagosome and kill and burst the immune cells by both hyphae-associated processes and physical forces: At early times after phagocytosis *C. albicans*'s growing hyphae together with proper cell surface exposure and architecture of the hyphae mediates early macrophage killing by activating a pro-inflammatory cell death, called pyroptosis, but after eight hours the elongation of hyphae cause death by piercing the immune cell (McKenzie, Koser *et al*. 2010, Uwamahoro, Verma-Gaur *et al*. 2014). This commensal fungus also shows tailored mechanisms against the host's immune barriers that environmental fungi, like *Aspergillus fumigatus*, do not have. For instance, *C. albicans* relies on diverse and versatile mechanisms to obtain iron from different host-specific sources, like heme or ferritin, whereas *A. fumigatus* uses a more generic siderophores system to acquire environmental iron (Schrettl, Bignell *et al*. 2004, Gerwien, Skrahina *et al*. 2018). In addition, upon copper deprivation (as an antimicrobial host strategy), *C. albicans* is the only microorganism reported so far to be able to swap cofactors and use manganese instead of copper for the detoxifying enzyme superoxide dismutase (Li, Gleason *et al*. 2015). These constitute clear examples of the adaptation of *C. albicans* to the human host, likely as a result of a commensal lifestyle during its evolution, which in turn has enabled the fungus to use these adaptions beyond the commensal framework and become pathogenic.

Intriguingly, the evolution of other commensal fungi has not followed the same direction, and although many have become opportunistic pathogens, they show different host adaptations and virulence potential. For example, *C. dubliniensis*, the phylogenetically closest species to *C. albicans* (Gilfillan, Sullivan *et al*. 1998, McManus, Coleman *et al*. 2008), is much less virulent despite being phenotypically very similar. Like *C. albicans*, *C. dubliniensis* is present in the normal oral flora of healthy individuals, but in the lower percentage of 3% compared to a 50% incidence of *C. albicans* (Chen C. and X. 2018). However, the percentage of *C. dubliniensis* is up to ten times higher in HIV-positive individuals (Coleman, Sullivan *et al*. 1997). Despite this, epidemiologic studies show twenty times less incidence of *C. dubliniensis* compared to *C. albicans* in systemic infections (Kibbler, Seaton *et al*. 2003, Odds, Hanson *et al*. 2007, Pfaller and Diekema

2007). Importantly, *C. dubliniensis* generally shows less filamentation than *C. albicans*. Genome comparison between two species shows a loss of several virulence-related genes in *C. dubliniensis* (Moran, Coleman *et al*. 2012). Evolutionary, it also did not expand important hyphae-associated gene families, considered to be involved in the host-pathogen interaction, which are important for *C. albicans* virulence (Moran, Coleman *et al*. 2012). Interestingly, *C. dubliniensis*, as well as *C. tropicalis* (another pathogenic *Candida* species) contain genes homologous to the *C. albicans ECE1* gene, which encodes the toxin candidalysin. Surprisingly, synthetic versions of candidalysin from these species are even more potent than candidalysin from *C. albicans* (Jonathan Richardson, personal communication). However, both species elicit less epithelial damage than *C. albicans*. The reason may be a lower *ECE1* expression (or Ece1 processing and secretion). This may be related to their poorer filament induction and hyphal maintenance, which may result in lower overall toxin production and, consequently, epithelial damage (Jonathan Richardson, personal communication). Alternatively, *ECE1* in these species is expressed in an entirely different context. All this points to a model where *C. dubliniensis* is undergoing reductive evolution as commensal adaptation to a niche in which virulence traits (like those of *C. albicans*) are not advantageous (Moran, Coleman *et al*. 2011). On the other side, virulence traits of *C. albicans* seem to provide the fungus a high advantage and adaptiveness specifically during infection: In 2014 Wartenberg *et al*. showed that a mutant of *C. albicans* which was incapable of forming hyphae evolved to recover the ability to form hyphae, and in turn regained its virulence, when co-incubated continuously with macrophages (Wartenberg, Linde *et al*. 2014). Evidently these phagocytes represented an environment where the specific virulence traits of *C. albicans* are required for its survival.

Nonetheless, virulence factors can become detrimental for the fungus even during infection when they are identified by the host and trigger strong antifungal immune responses. In this case they can become avirulence factors, since their recognition by the host reduces the progression of the pathogen (Lo Presti, Lanver *et al*. 2015, Siscar-Lewin, Hube *et al*. 2019). Candidalysin is one example of a factor with a dual property as a virulence and avirulence factor (Siscar-Lewin, Hube *et al*. 2019, Koenig, Hube *et al*. 2020). This toxin acts as a virulence factor when damaging epithelial cells and macrophages to allow the fungus to invade tissue and evade the immune system. It is an avirulence factor when the damage it generated promotes and activates antifungal immunity against the invading *C. albicans* in immunocompetent hosts (Moyes, Wilson *et al*. 2016, Verma, Richardson *et al*. 2017, Kasper, Koenig *et al*. 2018, Drummond, Swamydas *et al*. 2019). Other virulence factors, like the transporter Dur31, have been

targets during the host-pathogen co-evolution and are used by the host to the detriment of *C. albicans*. Dur31 is required for full virulence *in vitro* but also transports highly cytotoxic human antimicrobial peptides into the fungal cell, thereby committing *C. albicans* to a suicide-like process (Mayer, Wilson *et al*. 2012).

Thus, *C. albicans* has evolved towards a more virulent phenotype in contrast to its more commensal relative *C. dubliniensis*. The virulence traits have conferred *C. albicans* an adaptive advantage outside the commensal framework. This, however, comes with the price of being more "visible" to the host, who has learnt to counterattack *C. albicans*' most damaging virulence factor, thereby allowing a healthy host to keep the fungal virulence potential in check.

4.3.2 Environmental fungi

The ability of fungi to cause diseases has not only emerged as a result of host-fungus co-evolution. Environmental fungi, such as *Aspergillus*, *Cryptococcus, Coccidioides* or *Histoplasma* spp., also exhibit the "pathogenic requirements" that allow them to cause diseases. These virulence traits are thought to have evolved in the so-called "environmental virulence school", as these microorganisms encounter environmental conditions (changes in pH, high temperatures, phagocytosis by soil amoeba, etc) that are similar to the ones they face within the host (Bliska and Casadevall 2009, Kohler, Hube *et al*. 2017, Casadevall, Fu *et al*. 2019). Along with these adaptations, there are often genetic signs of evolution towards an animal-associated lifestyle, which similarly supports development of pathogenesis. A typical sign is the loss of genes that are non-adaptive in the new animal niche – making them antivirulence genes, since their function impairs host adaptation and virulence progression (Bliven and Maurelli 2012, Siscar-Lewin, Hube *et al*. 2019). One example of this can be found in *Coccidioides* spp., which are considered an emergent disease-causing fungal group due to their increased incidence as agents of infection in recent years (Hector, Rutherford *et al*. 2011). These species inhabit arid or semi-arid regions with alkaline soil and elevated average temperatures. Accordingly, the fungi can tolerate high temperatures up to 60°C. Compared to their non-pathogenic relatives, these fungi exhibit an increased number of keratinase-encoding genes, but have lost genes involved in plant matter digestion (a nutrient source in the environment). These keratinase enzymes provide nutrients to the fungus when growing as a mycelium on the animal carcass, then the fungus becomes accessible to the environment and spreads in the soil until encounters a new host (Del Rocio Reyes-Montes, Perez-Huitron *et al*. 2016).

The expansion of these genes is a clear sign of the close association and adaptation of

this fungus to animals during the evolution from a plant-saprophytic fungus to an animal pathogen (Sharpton, Stajich *et al*. 2009, Siscar-Lewin, Hube *et al*. 2019), a process in which the plant-degrading enzymes became antivirulence genes.

Another environmental opportunistic pathogen is *Cryptococcus neoformans*, a common cause of fungal meningoencephalitis in immunocompromised people, especially in those with HIV/AIDS. It is found as an environmental saprophyte, often in bird droppings (Kohler, Hube *et al*. 2017, Zaragoza 2019). As a result of adapting to these niches, including birds, *C. neoformans* is able to grow at a temperature of 37°C. Its main virulence factors are a glucurono- and galactoxylomannan capsule that blocks phagocytosis, and cell wall-associated melanin that confers resistance to many chemical and physical stresses (Zaragoza 2019). In addition to its capsule, this pathogen has sophisticated ways to survive phagocytosis. For instance, it is able to prevent phagosome acidification, detoxify the oxidative environment within the phagosome, and escape macrophages in a nonlytic manner (Ding, Festa *et al*. 2013, Kohler, Hube *et al*. 2017, Zaragoza 2019). These adaptations are thought to have evolved as a result of its interaction with environmental predator amoebae (Steenbergen, Shuman *et al*. 2001, Casadevall, Fu *et al*. 2019). Certain mutations occur frequently *in vivo*, *e.g*. in genes responsible for capsule organization and melanin production. These mutations influence the host inflammatory response and fungal stress resistance, respectively, giving rise to hypervirulent and persistent mutants of *C. neoformans*. As such, they are again good candidates for putative antivirulence genes in human pathogens (Siscar-Lewin, Hube *et al*. 2019).

Lastly, *Aspergillus* species are widespread in the environment but are usually found on decaying organic matter. *A. fumigatus* is one of the main causative agents of respiratory and systemic fungal infections (Sugui, Kwon-Chung *et al*. 2014). It shows a high thermotolerance that makes it resistant to human fever, as well as tolerance to a wide range of stresses, such as osmotic and oxidative stress, desiccation and starvation – all of them feature in accordance with its saprophytic lifestyle in soil (Paulussen, Hallsworth *et al*. 2017). *A. fumigatus* also produces gliotoxin as its most abundant mycotoxin, which is considered to have evolved and be produced as a secondary metabolite against environmental phagocytic amoebae (Hillmann, Novohradska *et al*. 2015). Nevertheless, gliotoxin also constitutes a virulence factor with suppressive properties against innate and adaptive immunity (Arias, Santiago *et al*. 2018). Another main virulence factor is cell wall melanin. It is present in *A. fumigatus* spores where it is involved in antigen masking, prevention of phagosome maturation, and protection against oxidative stress (Gomez and Nosanchuk 2003, Paulussen, Hallsworth *et al*. 2017). However, the virulence factor

melanin can turn into an avirulence factor in hosts that recognize it via a specific C-type lectin receptor and trigger a protective immune response. A single nucleotide polymorphism of this receptor was identified in humans to negatively affect the myeloid inflammatory responses and increase the susceptibility of stem-cell transplant recipients to disseminated *Aspergillus* infections (Stappers, Clark *et al*. 2018). This, therefore, serves as another example of the potential dual properties of fungal virulence factors, which can confer virulence or avirulence functions depending on host adaptations, conditions and responses. Interestingly, in this case the host has evolved to recognize and develop a counterattack against the environmental fungus *A. fumigatus* despite the lack of ongoing commensal interaction (Siscar-Lewin, Hube *et al*. 2019).

Therefore, environmental fungi show pathogenicity traits which are analogous to commensal fungi. These have in part emerged or have been enhanced by the loss of antivirulence genes in the fungi. The presence of avirulence genes in environmental fungi shows that the host adapted to the pathogen's virulence traits, which in itself is an indication of host-pathogen co-evolution, likely due to intermittent contact.

4.4 *Candida glabrata*

4.4.1 Importance of *C. glabrata* as a fungal pathogen

Candida glabrata is considered a commensal of the human mucosa and its incidence as an opportunistic pathogen has been increasing in the last forty years. It has risen to become the most prominent non-*Candida albicans Candida* (NCAC) species to cause disease (Hazen 1995, Fidel, Vazquez *et al*. 1999, Rodrigues, Silva *et al*. 2014). Nowadays, this fungus constitutes about one-third of the isolates from candidemia cases in the US, and its incidence is also steadily increasing in European countries and in Australia (Lamoth, Lockhart *et al*. 2018). Besides the risk factors mentioned above for commensal fungal infections, elderly people are especially at risk of *C. glabrata* infections (Perlroth, Choi *et al*. 2007, Pfaller and Diekema 2007). Even though the clinical importance of this fungus is increasing, *C. glabrata*'s pathogenicity strategies differ to a large extent from *C. albicans* and still remain poorly understood (Brunke and Hube 2013). Important scientific efforts have focused on understanding the evolutionary emergence of virulence traits in *C. glabrata* through genomic and metabolic studies.

Since 2004, when the *C. glabrata* genome became available, genomic sequence comparisons of the brewer's yeast *Saccharomyces cerevisiae* and *C. glabrata* have been performed, as it was shown that phylogenetically *C. glabrata* was more closely related to *S. cerevisiae* than to *C. albicans* (Dujon, Sherman *et al*. 2004, Roetzer, Gabaldon *et*

al. 2011). Furthermore, later phylogenetic studies showed that *C. glabrata* belongs to the *Nakaseomyces* genus, which contains three environmental species (*Nakaseomyces delphensis*, *Candida castellii* and *Nakaseomyces bacillisporus*) (Kurtzman 2003) and two milder pathogens (*Candida nivariensis* and *Candida bracarensis*) in addition to *C. glabrata* (Alcoba-Florez, Mendez-Alvarez *et al*. 2005, Correia, Sampaio *et al*. 2006). Recent work has compared the genomes of the *Nakaseomyces* genus and changed the view of the emergence of virulence in *C. glabrata* (Gabaldon, Martin *et al*. 2013, Gabaldon and Carrete 2016). These new results show that what were considered specific pathogenic traits of *C. glabrata* in comparison to *S. cerevisiae*, are in fact events that the whole genus, environmental and pathogenic species, underwent. Among these traits are an optimal growth at temperatures close to 37°C and certain auxotrophies, such as nicotinic acid, pyridoxine and thiamine, which have been suggested to be a result of niche-specific host adaptation (Casadevall 2008). Importantly, Gabaldón *et al*. also showed the independent expansion of virulence-related genes, such as the *EPA* gene family (Epithelial adhesins) and the presence of *YPS* tandem arrays (Aspartyl proteases), only in the pathogenic species, and here especially in *C. glabrata* (Gabaldon, Martin *et al*. 2013).

Furthermore, four genes were identified in the lineage of *C. glabrata* that show signatures of positive selection in form of accelerated evolutionary rates (*dN*/*dS* ratio >1) (Gabaldon, Martin *et al*. 2013). It has been suggested that the selection pressure that acted or still acts on these genes may be related to a new environment to which *C. glabrata* was or is adapting to – for instance, the human host. Lastly it has been speculated that the ability for commensalism and pathogenesis in the *Nakaseomyces* genus may have emerged from an environmental ancestor that came into frequent contact with the mammalian gut. Such interactions may have given rise to species that can persist in such environment and develop further adaptations (Gabaldon, Martin *et al*. 2013). Based on this, in 2019 Gabaldón and Fairhead questioned the assumption that *C. glabrata* is a natural commensal of the human mucosa, and alternatively suggested it to be a nomadic opportunistic commensal (Gabaldon and Fairhead 2019). Evidences that support such a claim are the isolation of *C. glabrata* from different, unrelated environments (coffee beans, cloaca swabs of several bird species, mobile phones), large diversity between clades, and a lack of geographic correlation of the genotypes of *C. glabrata* clinical isolates (Carrete, Ksiezopolska *et al*. 2018). All in all, whether the human is a natural niche for *C. glabrata* or not, this fungus clearly possesses the tools which enabled it to become one of the most relevant fungal opportunistic pathogen of humans.

4.4.2 Pathogenic traits of *C. glabrata*

C. glabrata is a haploid fungus that does not form hyphae and elicits much less damage and inflammation than *C. albicans* during interaction with host cells *in vitro* and in mice*,* but is still able to disseminate and invade host tissues (Jacobsen, Brunke *et al*. 2010, Jacobsen, Grosse *et al*. 2011, Atanasova, Angoulvant *et al*. 2013). This fungus relies on numerous adhesins-encoding genes, the *EPA* genes for epithelial adhesion and biofilm formation. For these reasons, they are considered to be one of its main virulence factors (Cormack, Ghori *et al*. 1999, De Las Penas, Pan *et al*. 2003, Kojic and Darouiche 2004, von Eiff, Jansen *et al*. 2005). For dissemination without hyphae (which in many fungi are the tissue-penetrating structures), it has been suggested that *C. glabrata* relies on endocytosis or epithelial disruption by action of humans (catheters) or other microbes (*e.g*. epithelial disruption by *C. albicans*'s hyphae) (Perlroth, Choi *et al*. 2007, Coco, Bagg *et al*. 2008). Furthermore, *C. glabrata* triggers only a low pro-inflammatory response, where the only significantly induced cytokine is GM-CSF both *in vitro* and *in vivo* (Jacobsen, Brunke *et al*. 2010, Seider, Brunke *et al*. 2011). This cytokine is responsible for the activation and recruitment of macrophages. Interestingly, although *C. glabrata* is recognized and engulfed by these immune cells (Seider, Brunke *et al*. 2011), viable yeasts are able to arrest phagosome maturation and acidification. Then, *C. glabrata* can duplicate within the phagosome until escaping the phagocyte by bursting free, which is considered another of its main pathogenic traits (Seider, Brunke *et al*. 2011, Kasper, Seider *et al*. 2014, Kasper, Seider *et al*. 2015, Sprenger *et al*. 2018).

The cell surface-associate proteases encoded by the *YPS* gene family seem to be involved in preventing macrophage activation, since their deletion mutants trigger strong macrophage activation and killing of the fungus (Kaur, Ma *et al*. 2007). The fungus is also able to largely suppress the production of ROS in the phagosome, and it possesses a catalase, Cta1 that is more active than those of *C. albicans* and *S. cerevisiae* and that may provide the fungus with a high tolerance to ROS within the phagosome (Cuellar-Cruz, Briones-Martin-del-Campo *et al*. 2008). Nevertheless, it has been shown that ROS may not act alone in killing the fungus. This seems to happen rather due to its combination with additional stresses, since experimental suppression of ROS by macrophages did not increase the fungal survival (Kasper, Seider *et al*. 2015). Within the phagosome this yeast also seems to rely on carbon sources other than glucose, activating the glyoxylate cycle, gluconeogenesis, and autophagy to survive and replicate independent of readily accessible glucose (Rai, Balusu *et al*. 2012, Fukuda, Tsai *et al*. 2013, Seider, Gerwien *et al*. 2014, Chew, Brown *et al*. 2021). In this context many efforts

have been taken to elucidate how the assimilation of available carbon source affects yeast growth, immune-evasion, survival and persistence within the macrophages; antifungal resistance and biofilm formation (Ene, Cheng *et al*. 2013, Brown, Brown *et al*. 2014, Mota, Alves *et al*. 2015, Chew, Ho *et al*. 2019).

The accumulation of trehalose, a simple non-reducing sugar containing two glucose subunits, could also play a role in the survival of *C. glabrata* within macrophages, as it has been shown that this molecule does not only serve as a carbon source, but also as an intracellular and extracellular protectant against different stresses (Pedreno, Gonzalez-Parraga *et al*. 2007, Sanchez-Fresneda, Martinez-Esparza *et al*. 2014, Eleutherio, Panek *et al*. 2015, Sanchez-Fresneda, Guirao-Abad *et al*. 2015). This carbohydrate interacts with proteins and lipids of the membrane structures to bolster them against oxidation and denaturation from inside and outside, in conditions of dissecation, oxidation, and high temperatures (Elbein, Pan *et al*. 2003). Trehalose is exclusively synthesized by bacteria, fungi, and plants, and it has been shown to have a biological function as an anti-stress agent during infection for different species of human pathogenic fungi (Alvarez-Peral, Zaragoza *et al*. 2002, Van Dijck, De Rop *et al*. 2002, Alcoba-Florez, Mendez-Alvarez *et al*. 2005).

Another very important pathogenic trait of *C. glabrata* is its high intrinsic resistance to azole antifungals (Ostrosky-Zeichner, Rex *et al*. 2003) and the rapid development of resistance to even higher concentrations of azoles (Warnock, Burke *et al*. 1988, Hitchcock, Pye *et a*l. 1993, Sanglard, Ischer *et al*. 1999, Bennett, Izumikawa *et al*. 2004, Pfaller, Diekema *et al*. 2004, Tsai, Krol *et al*. 2006). Azoles inhibit the enzyme lanosterol demethylase, the product of the *ERG11* gene, which is involved in ergosterol synthesis. As a result, there is an alteration of the structure and function of the cell membrane, which causes an arrest of fungal growth. In combination with the antifungal immune response, this will result in clearance of the fungus (Ghannoum and Rice 1999). Fluconazole is the main antifungal used in cases of candidemia and refractory infection, mainly due to its low price and low cytotoxicity. However do to the intrinsic and rapid emergence of *C. glabrata* resistance to this drug, the use of other drugs, such as voriconazole, echinocandins or amphotericin B is now advised as a first-line treatment for *C. glabrata* infections (Pappas, Kauffman *et al*. 2016).

The most common azole resistance mechanism found in *C. glabrata* is the activation of the transcription factor Pdr1. This in turn triggers the constitutive expression of the ABC transporters-encoding genes *CDR1* and *CDR2*, which confer azole resistance by export of the drug (Sanglard, Ischer *et al*. 2001, Vermitsky, Earhart *et al*. 2006, Whaley and

Rogers 2016). Gain-of-function (GOF) mutations in the *PDR1* gene account for the majority of cases of acquired azole resistance reported so far (Sanguinetti, Posteraro *et al*. 2005, Ferrari, Ischer *et al*. 2009, Whaley and Rogers 2016, Whaley, Zhang *et al*. 2018). Moreover, the activation of Pdr1 not only acts on the expression of efflux pump genes but also on other genes involved in multidrug resistance, including the *EPA* genes, lipid metabolism-related genes, cell wall structure genes, and stress response and Golgi-ER-related genes (Vermitsky, Earhart *et al*. 2006). Furthermore, a high expression level of *CDR1* was shown to be responsible for increased fungal burden and thus virulence in a mice model of infection, although the mechanism remains unknown (Ferrari, Sanguinetti *et al*. 2011). In addition to *PDR1* activation by GOF mutations, azole resistance has been reported to occur in *C. glabrata* and *S. cerevisiae* after loss of mitochondrial function, mainly due to complete (rho^0) or partial loss (or presence of deleterious mutations) (rho^-) of mitochondrial DNA (mtDNA) (Lipinski, Kaniak-Golik *et al*. 2010). Yeasts of both species continue to be viable after this loss and grow as small colonies, earning them the name of *petite*-positive yeasts (Goldring, Grossman *et al*. 1970, Chen and Clark-Walker 2000, Sanglard, Ischer *et al*. 2001), unlike other yeast, such as the opportunistic pathogens *C. albicans* and *C. neoformans* (Chen and Clark-Walker 2000, Toffaletti, Nielsen *et al*. 2004).

The *petite* phenotype is characterized by small colonies, long generation times, and lack of growth in alternative carbon sources (reviewed by Day 2013). Additionally, the loss of mtDNA triggers changes in the mitochondrial membranes leading to diminished membrane association of Psd1, the phosphatidylserine decarboxylase, in the mitochondrial inner membrane (Gulshan, Schmidt *et al*. 2008). This diminished association triggers the activation of the transcriptional factor Pdr1 and subsequently *PDR1*-dependent efflux pumps activation and azole resistance (Hallstrom and Moye-Rowley 2000, Traven, Wong *et al*. 2001, Zhang and Moye-Rowley 2001, Gulshan, Schmidt *et al*. 2008; Bouchara *et al*., 2000; Brun *et al*., 2003; Brun *et al*., 2004; Ferrari *et al*., 2011; Kaur, Castano, & Cormack, 2004; Tsai *et al*., 2006). The *petite* phenotype can be induced by incubation with high concentrations of ethidium bromide or azole antifungals (Goldring, Grossman *et al*. 1970, Bouchara, Zouhair *et al*. 2000, Sanglard, Ischer *et al*. 2001): Ethidium bromide inhibits mtDNA synthesis and degrades the existing mtDNA (Goldring, Grossman *et al*. 1970), while the exact mechanism how azoles trigger mitochondrial dysfunction is not clear. Inhibition of synthesis or degradation of mtDNA by azoles has not been reported yet, and azoles are rather connected to a temporary loss of mitochondrial function (Kaur, Castano *et al*. 2004).

Very few *petite* mutants of *C. glabrata* have been isolated from patients with candidiasis (Peng, Dong *et al*. 2012), and of those some were undergoing fluconazole treatment (Bouchara, Zouhair *et al*. 2000, Posteraro, Tumbarello *et al*. 2006). Only one of these *petite* clinical isolates has been further characterized, and it was shown to be more virulent in animal models (Ferrari, Sanguinetti *et al*. 2011). However, when the isogenic respiratory-competent strain from an earlier isolation was made *petite* by ethidium bromide treatment, it showed decreased virulence. Another study reported that an ethidium bromide-induced *petite* showed much lower virulence in animal models than the parental strain (Brun, Dalle *et al*. 2005). Thus, the clinical relevance of these mutants is still controversial. The slow growth of *petite* mutants limits their identification in the clinics, and consequently the investigation of the possible implications of this phenotype in *C. glabrata*'s adaptation to the host and virulence.

4.5 Mitochondria and pathogenicity

The aerobic respiratory chain is a metabolic process through which ATP is formed. The electron transport chain is made up of a series of complexes (normally four: I, II, III, IV), that transfer electrons from electron donors to electron acceptors via redox reactions, and couples this electron transfer with the transfer of protons across a membrane. In aerobic respiration, the flow of electrons finishes in the cytochrome c oxidase (Complex IV) where molecular oxygen is used as the final electron acceptor. Although it is not an essential pathway for many microorganisms to survive, it provides the most efficient energy production from nutrient oxidation and is used by both prokaryotes and eukaryotes. If this process is blocked in heterotrophic organisms, their energy demand can only be met by fermentable carbon sources. Then, the more inefficient fermentation can result in slow-growing colonies with a small size, called small colony variants (SCV) in bacteria and *petite* mutants in yeasts (Goldring, Grossman *et al*. 1970, Goldring, Grossman *et al*. 1971, Proctor, von Eiff *et al*. 2006, Day 2013). Although these mutants are considered physiologically impaired due to their slow growth and metabolism, they show other physiological changes that affect the host-pathogen interplay and can become adaptive during pathogenesis (Day 2013). A good example can be found with the important intracellular pathogens *Staphylococcus aureus* and *Salmonella enterica* serotype Typhimurium that are known to persist in normally lethal environmental conditions, forming SCVs. These SCVs show a decreased antibiotic susceptibility, and are linked to chronic and relapsing, often therapy-refractory infections (Proctor, von Eiff *et al*. 2006). Moreover, they show reduced expression of virulence factors, but higher adhesion, which promotes internalization in host cells and facilitate immune-evasion and

long-term persistence within their hosts (Proctor, von Eiff *et al*. 2006, Johns, Purdy *et al*. 2015, Kahl, Becker *et al*. 2016). As a result, SCVs from many gram-positive and -negative bacteria have been recovered from clinical tissues, including abscesses, blood, bones and joints, the respiratory tract, and soft tissues (Proctor, von Eiff *et al*. 2006, Kahl, Becker *et al*. 2016). The emergence of SCVs has been reported to happen due to defects in the electron transport chain, mainly as a result of mutations in the synthesis pathways of the intermediates of the electron transport chain components, such as menadione or haem (Kohler, von Eiff *et al*. 2003, von Eiff, Jansen *et al*. 2005, Seggewiss, Becker et al. 2006). Therefore, when these compounds are supplemented, SCVs are still able to switch back to respiratory metabolism (Proctor, von Eiff *et al*. 2006). It has been proposed that SCVs may contribute to bacterial heterogeneity, which might confer an adaptive advantage upon environmental changes (Day 2013, Tuchscherr, Pollath *et al*. 2019).

For fungi, *S. cerevisiae* and *C. glabrata* are so far the only known *petite*-positive yeast species with importance to humans (Chen and Clark-Walker 2000). In addition to the pleiotropic drug resistance, the *petite* phenotype of the baker's yeast has been found to be more resistant to heat shock and stress, which is correlated with an increased life span (Kennedy, Austriaco *et al*. 1995, Traven, Wong *et al*. 2001). Only *C. glabrata petite* mutants have been (rarely) found in infections (Bouchara, Zouhair *et al*. 2000, Posteraro, Tumbarello *et al*. 2006), and so far only one *petite* mutant has been published to possess any pathogenic advantage although a direct connection of its virulence to the *petite* phenotype could not be shown (Ferrari, Sanguinetti *et al*. 2011). Analogous to the SCVs, these strains have been isolated from patients under antifungal treatment (Bouchara, Zouhair *et al*. 2000) and with recurrent fungemia (Posteraro, Tumbarello *et al*. 2006). Moreover, the single *C. glabrata petite* strain that has been further characterized showed decreased susceptibility to antifungals and increased fungal burden in animal models of infection (Ferrari, Sanguinetti *et al*. 2011).

In yeast the *petite* phenotype can result from two major types of mitochondrial dysfunction: Mutations that affect the function the electron transport chain (components of the electron transport chain, ATP synthase complex, heme biosynthesis or fatty acid metabolism, which affect mitochondrial membrane integrity), or a total or partial loss of mtDNA (Contamine and Picard 2000). *Petites* can arise spontaneously, and many decades ago the frequency of this event was reported as 0.1-1% for *S. cerevisiae* (Chen and Clark-Walker 2000). This number can be increased by incubation in glycerol, with heat, light, low concentrations of antibiotics (which inhibit protein synthesis), and with membrane-active agents, like phenyl alcohol or isopropanol (reviewed by Day 2013). The stability of the *petite* phenotype is not yet clear, as some studies using antibiotics or

azoles showed a reversion of *petite* of *C. glabrata* to wild type when the drug was removed (Williamson, Maroudas *et al*. 1971, Kaur, Castano *et al*. 2004). Thus, the environmental or clinical significance of the *petite* phenotype is not known yet. Similarly, whether there are selective pressures or genetic predispositions for the appearance of such mutants in the host or the environment remains unknown.

5. Aims of this study

Fungal infections are becoming more and more prevalent worldwide (Bongomin, Gago *et al*. 2017). Learning how pathogenic fungal species develop their host-adaptive mechanisms and further their pathogenicity is crucial to understand the emergence and evolution of virulent species. With this aim, Manuscript I introduces the concepts of antivirulence and avirulence genes in human fungal pathogens, based on the well-known antivirulence genes in bacterial pathogens and avirulence genes in the plant fungal pathogens. The specific adaptations of different *Candida* yeasts to the human host are investigated in Manuscript II as examples for such a fungal-host co-evolution. Indeed a species-specific response of the most common *Candida* pathogens can be shown in response to contact with host epithelial cells, while the epithelial cells exhibit a pro-commensal response to all of these fungi in absence of damage.

C. glabrata represents one of the most important causes of candidiasis (Enoch, Yang *et al*. 2017). The lack of epithelial damage and inflammatory response, the ability to survive and replicate within the phagosome, and the high intrinsic resistance to stress and antifungals, has made stealth and persistence the main pathogenic traits of this fungus (Brunke and Hube 2013, Rodrigues, Silva *et al*. 2014, Kasper, Seider *et al*. 2015). In Manuscript III we show that *C. glabrata* additionally relies on the bed-hedging strategy of *petite* phenotype that allows this commensal yeast to succeed as a pathogen – something that would otherwise be considered a loss of functionality. Despite showing a decrease in fitness, cells with the *petite* phenotype are able to resist high concentrations of azole antifungals and survive better within the macrophages at early time points. Furthermore, such a strategy shows a cross-resistance effect, since *C. glabrata* turns *petite* upon exposure both to azoles and to phagocytosis, showing for the first time the interplay between antifungal resistance and immune evasion in a human pathogenic fungus. Importantly, there are indications for a clinical relevance of this growth phenotype, as *petites* are found among clinical isolates.

In a further investigation of phagocyte-specific adaptations, this study also elucidates relevant pathways for the survival and persistence of *C. glabrata* within macrophages. On the one hand, the trehalose known to accumulate in yeasts serves as protection against oxidative stress as well as a carbon source after stress exposure (Eleutherio, Panek *et al*. 2015). Deletion mutants of the putative trehalase enzymes of *C. glabrata* in Manuscript IV show a decrease in virulence and their mid- to long-term survival of

phagocytosis, thus uncovering a new important pathway involved in adaptation of *C. glabrata* to the host's immune response. On the other hand, replication of *C. glabrata* in the phagosome has been attributed to the utilization of alternative carbon sources (Kasper, Seider *et al*. 2015). However, the use of certain carbon sources, like acetate, seems to be rather detrimental to the evasion and survival of *C. glabrata* upon exposure to macrophages (Mota, Alves *et al*. 2015). The findings in Manuscript V demonstrate that the absence of acetate transporters benefits long-term survival of *C. glabrata* within macrophages, shedding light on the metabolic pathways involved in replication and persistence of *C. glabrata* within these immune cells and raising the possibility that these transporters act as antivirulence factors in the specific context of the phagosome.

6. Manuscripts

6.1 Manuscript I

Antivirulence and avirulence genes in human pathogenic fungi

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Virulence. 2019; 10(1): 935–947. Published online 2019 Nov 11. DOI: 10.1080/21505594.2019.1688753

Summary:

A range of fungi can cause superficial to systemic diseases in humans. This review introduces new concepts and gives an overview over the evolutionary mechanisms that led to the emergence of fungal pathogenicity and virulence. It focuses on the loss of non-adaptive genes in the host niche, named antivirulence genes, and those that impairs fungal progression within the host, the so-called avirulence genes. This review shows the analogy, to some extent, between the well-known antivirulence genes in pathogenic bacteria and the avirulence genes in phytopathogenic and even human-pathogenic fungi. Thus, this review introduces new concepts which are so far nearly unknown in the field of human pathogenic fungi, to broaden our view of human-fungal interplay and better understand the emergence and evolution of virulence in human pathogens.

The candidate is:
☒ First author ☐ Second author ☐ Corresponding author ☐ Coauthor

Estimated authors' contributions:

Author	Conception	Data analysis	Experimental	Writing	Provision of the material
SSL	70%			70%	
BH	10%			10%	
SB	20%			20%	

VIRULENCE
2019, VOL. 10, NO. 1, 935–947
https://doi.org/10.1080/21505594.2019.1688753

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REVIEW ARTICLE

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Antivirulence and avirulence genes in human pathogenic fungi

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ABSTRACT

Opportunistic commensal and environmental fungi can cause superficial to systemic diseases in humans. But how did these pathogens adapt to infect us and how does host-pathogen co-evolution shape their virulence potential? During evolution toward pathogenicity, not only do microorganisms gain virulence genes, but they also tend to lose non-adaptive genes in the host niche. Additionally, virulence factors can become detrimental during infection when they trigger host recognition. The loss of non-adaptive genes as well as the loss of the virulence potential of genes by adaptations to the host has been investigated in pathogenic bacteria and phytopathogenic fungi, where they are known as antivirulence and avirulence genes, respectively. However, these concepts are nearly unknown in the field of pathogenic fungi of humans. We think that this unnecessarily limits our view of human-fungal interplay, and that much could be learned if we applied a similar framework to aspects of these interactions. In this review, we, therefore, define and adapt the concepts of antivirulence and avirulence genes for human pathogenic fungi. We provide examples for analogies to antivirulence genes of bacterial pathogens and to avirulence genes of phytopathogenic fungi. Introducing these terms to the field of pathogenic fungi of humans can help to better comprehend the emergence and evolution of fungal virulence and disease.

ARTICLE HISTORY
Received 3 April 2019
Revised 22 August 2019
Accepted 1 October 2019

KEYWORDS
Fungal virulence; antivirulence; avirulence; host-pathogen interactions; evolution of virulence

Introduction

Diseases caused by pathogenic fungi are still frequently underestimated [1] but are more and more recognized as an important threat especially to immunocompromised populations. Currently, treatment of fungal infections in humans is limited to the use of a few classes of antifungal drugs [2]. Understanding the pathobiology of these fungi is essential to develop novel therapeutic approaches to extend our options to treat fungal infections. Diseases caused by fungi come in many forms: Dermatophytes affect approximately one fifth of the world population but are restricted to cause infections of skin, hairs, and nails. In contrast, *Candida*, *Aspergillus*, *Cryptococcus*, *Coccidioides*, *Pneumocystis* species, and *Histoplasma capsulatum*, are among the most important fungi that are able to cause diseases ranging from superficial to systemic [3]. Whereas they mainly affect immunocompromised hosts, a few environmental species are primary pathogens and can also cause disease in healthy individuals [3]. *Candida* spp. are among a small group of fungal species that are thought to have been commensal members of our microbiota for much of human evolution [3–7]. Likely because of this coevolution, these species have developed an impressive range of adaptations to the human environment, which allow the fungus to obtain nutrients, survive to host immunity, and withstand stress conditions within the human host – all of which is not only required for commensalism, but also a pre-requisite for pathogenicity [8–12].

Other human pathogenic fungi, although having evolved as saprophytes in the environment or in close relationships with birds and bats (like *Cryptococcus neoformans* and *H. capsulatum*) or rodents (like *Coccidioides* species) often exhibit infection strategies strikingly similar to the human commensal *Candida* species, from immune evasion to hydrolytic enzymes and toxins [13,14]. In fact, these convergent evolved strategies frequently resemble the mechanisms used to resist environmental phagocytes, like amoebae [15]. It has been suggested that an "environmental virulence school" allowed them to become successful human pathogens [3], as the same mechanisms allow them to resist, shield themselves, counteract and manipulate host immune responses [13]. Such training grounds for host interactions, commensal and environmental, might thereby explain the appearance of virulence factor genes in human pathogenic fungi.

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However, during the evolution of pathogenicity, fungi must also shed certain genes which are involved in "energy wasting" processes and have no selective advantage in the host or even trigger detrimental host responses. Such non-adaptive genes, known also as antivirulence genes, are very well described in bacterial pathogens [16]. In fact, the evolution toward pathogenicity of some very important infectious microorganism, such as *Yersinia pestis, Shigella* or *Francisella tularensis* (subsp. *tularensis* and *holarctica*), has been characterized by loss of genes [16,17]. Moreover, not all virulence factors are solely beneficial to the pathogen during interactions with the host. Virulence factors and their associated damage can also make the pathogen "visible" to the host's immune surveillance and therefore become disadvantageous [18,19]. Such factors have been studied in pathogenic fungi of plants, which are able to trigger a hypersensitive immune response in the infected plant and thereby promote plant resistance [20]. This interaction renders the pathogen avirulent, and genes encoding these effectors are consequently known as avirulence genes [20,21]. We propose that similar events take place in human-fungal interactions, and that we can analogously identify antivirulence and avirulence genes in fungal pathogens of humans. Thinking in these terms may help us to better understand the host-pathogen interplay, and eventually help us in the search for new fungal therapeutic targets to combat fungal infections.

The concept of antivirulence gene in human pathogenic fungi

To define antivirulence in fungi, we need first to have – for this review – a working definition of virulence for these organisms. It is now textbook knowledge that, while pathogenicity refers to the ability of a microbe to cause damage in a host *per se*, virulence is defined by the degree of damage the microbe can elicit (Box 1). This damage manifests as the interruption of normal host function (and usually tissue structure) at any of the cellular, tissue or organ levels, which as clinical manifestation is called disease [22,23].

Virulence itself can derive from direct action of the microbe, and classically virulence factors are defined as properties of the pathogen that, when deleted, impair their damage potential in the host but not their general viability (which is supported by virulence-independent, broad physiological factors) [23]. Examples of such virulence factors are the capacity to attach to or invade into host tissue (or both), as well as avoidance of host detection, inhibition of phagocytosis, and intracellular survival [24]. Even without dedicated virulence factors, microbial growth and persistence within the host can eventually induce damage through the host inflammatory response, and the virulence of some organisms is intrinsically linked to the ability of inducing a host inflammatory response that results in tissue damage [18,19,25]. On the other hand, even microbial virulence factors can only affect susceptible hosts, as exemplified by the many fungal pathogens which as opportunists do rarely affect an otherwise healthy host [3]. Therefore, the virulence of a microbe is evidently not solely dependent on microbial attributes but is rather determined by all of the host–microbe interactions. This requires us to extend on the previous concept of virulence, which explicitly excluded "physiological factors" that are essential for microbial growth. Such a more inclusive virulence factor definition then includes all microbial attributes that mediate host damage (Figure 1): from those essential for invasion to those essential for microbial growth within the host and a potential self-damaging host reaction [24]. Consequently, attributes that actively impair microbial fitness within the host or trigger an appropriate (i.e. not self-damaging) host recognition and response would be detrimental to the pathogen.

How virulence emerged across the human pathogenic fungi is still far from understood. One strategy to investigate the emergence of pathogenicity focusses on the comparison of genomes from the most closely related nonpathogenic and pathogenic species. This includes the study of past genomic re-arrangements, lineage-specific gene duplications, mutations and indicators of positive selection. For *Candida* species, the main mechanisms identified so far to promote pathogenicity are total or partial chromosomal rearrangements, gene duplication and loss, gene family expansion, and inter-species hybridization [26]. In *A. fumigatus*, gene duplication and diversification in genomic islands is a known mechanism to acquire novel genes, which are absent in the

Box 1. Definitions of concepts.

Pathogenicity: the ability of a microbe to cause damage to a host.

Virulence: the degree of damage a microbe can elicit. This damage manifests as the interruption of normal host function (and usually tissue structure) at any of the cellular, tissue or organ levels, which as clinical manifestations is called disease.

Virulence factor: a microbial attribute that causes damage to a host, either by direct action or indirectly by the host response or by allowing growth and survival when facing the host reaction (the latter is often also called a virulence determinant). The deletion of a virulence factor gene usually reduces virulence.

Antivirulence factor: a microbial attribute that reduces the direct or indirect damage to the human host. The gene that encodes an antivirulence factor is an **antivirulence gene**, and its deletion or loss leads to an increased virulence.

Avirulence factor: a virulence factor that is recognized by a host specific receptor, which triggers a host immuno response that concludes with the pathogen's virulence. The gene that encodes an avirulence factor is an **avirulence gene**.

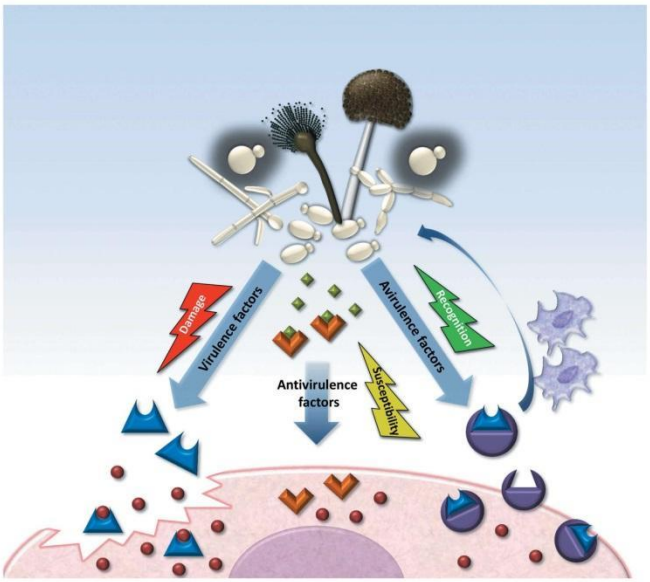

Figure 1. Illustration of virulence, antivirulence, and avirulence factors and their adaptive consequences within the host. Fungal factors expressed during host–pathogen interactions can lead to three different outcomes. From the pathogen's perspective, a virulence factor (blue form) can be advantageous to overcome the host immune barrier, invade, or withstand stress conditions during infection. An antivirulence factor, in contrast, might be advantageous outside the host (green squares), but has a detrimental effect within the host, since it lowers the pathogen's fitness, immune evasion ability or stress resistance. Lastly, a potential virulence factor can lose its function and become detrimental to the pathogen when the host develops specific receptors (purple form). If these recognize the factor or its action in the host, it can trigger an (immune) response that stops the progression of infection and turns the virulence factor into an avirulence factor.

nonpathogenic relatives and mainly encode secondary metabolites, such as mycotoxins [27,28]. Thus, these gene factories might become the source of new virulence factors in *A. fumigatus*. Clear examples of evolution toward pathogenicity are found in the genomes of different *Candida* species. It has been shown that genes encoding virulence-associated adhesins, like the *ALS* and *EPA* families of *C. albicans* and *C. glabrata*, respectively, or hydrolases like the *C. albicans SAP* or *LIP* families multiplicated in these pathogenic species [29,30]. In contrast, their loss has occurred in related yeasts: *C. dubliniensis* has lost, for example, *ALS3*, an important virulence gene in *C. albicans* [28]. Similarly, the nonpathogenic relative of *C. glabrata, Nakaseomyces delphensis*, has one single copy of the *EPA* genes whereas *C. glabrata* possesses 18 [29]. Finally, the causative agents of valley fever, *Coccidioides* spp. (*Coccidioides immitis* and *Coccidioides posadasii*), show enrichment in keratinase-encoding genes, a sign of their close association with keratin-rich animals during the evolution from a plant-saprophytic fungus to an animal pathogen [31].

For a microbe in contact with a host, the lack of nutrients, generally harsh environmental conditions and the need to evade immune defenses [12,14,32] can exert selective pressures toward the evolution of a virulent phenotype. Positively, this can be the acquisition of genes that encode virulence factors by gene transfer [33–35] or mutations as described above, but the loss of certain genes has been recognized more and more as an important event for the emergence of virulence in bacterial species [16,17]. These antivirulence genes (Figure 1), whose expression is largely or absolutely incompatible with an at least transient pathogenic lifestyle, are classically present and active in the genomes of nonpathogenic antecessor, but become pseudogenes or lost in pathogenic species [16]. Bliven et al. explicitly exclude genes that are active in the majority of wild-type

strains of a species, but inactive in virulent strains, and call these regulators or suppressors of virulence instead. Per definition, the insertion of active antivirulence genes into derived pathogenic strains leads to a decrease in their virulence. Examples have been found in bacterial pathogens, such as *Shigella, Salmonella*, and *Yersinia* species: In contrast to its nonpathogenic antecessor *Escherichia coli, Shigella* species have lost the ability to synthesize *de novo* nicotinic acid (NAD) by inactivation of the genes *nadA* and *nadB* [36, 37]. It was shown that the pathway intermediate quinolinic acid inhibits the type III secretion system of *Shigella* spp., and thus its virulence. *Shigella* spp. instead imports exogenous nicotinic acid, and the introduction of the biosynthesis genes reduces their virulence – marking these genes as antivirulent. *Yersinia pestis*, in contrast to its milder enteric human pathogen antecessor *Y. pseudotuberculosis*, has lost metabolic and motility associated genetic loci for a successful colonization of the mammalian gastrointestinal tract and become a systemic pathogen that invades the lymphatic system. In fact, the gene loss experienced by *Y. pestis* comprises a considerably larger proportion of the genome than what has been acquired by gene gain events in the pathogenic *Y. pestis* lineage [38].

Unlike in pathogenic bacteria, to our knowledge no antivirulence genes have been explicitly named in human pathogenic fungi. However, we can find examples of pseudogenization and loss of genes accompanying the evolution toward both commensalism and pathogenicity in fungi [28]. In addition, hypervirulence as a result of experimental gene inactivation is frequently observed [39]. This indicates that loss of function is a possible evolutionary trajectory to increased virulence also in the human host. With this background, we will now look into possible antivirulence genes in pathogenic fungi. If we follow the very strict definition of antivirulence genes from Bliven and Maurelli [16], which requires both, avirulent antecessors and virulent descendant species, we would have to exclude from our investigation those genes that are absent or inactive in virulent strains, but active in nonpathogenic "wild type" strains of the same species. However, this makes antivirulence a property of the gene which is mainly dependent on the definition of species and the classification of wild types *vs.* derived strains. For the sake of this review, we will therefore include examples of genes which exert their antivirulent properties in nonpathogenic strains, but whose loss can be associated with a significant increase in virulence in clinical isolates or experimentally generated mutants. We thereby unlink the genes' property from phylogenetic and epidemiological considerations.

Potential antivirulence genes of human pathogenic fungi

Antivirulence genes identified from pathogen evolution

The adaptation of a microorganism to a new environment requires the loss of non-adaptive genes in order to optimize the energy expenses in the new niche. *C. glabrata* has lost several metabolic pathway genes compared to the generally nonpathogenic yeast *Saccharomyces cerevisiae* [40]. These losses include genes of the galactose metabolism, nitrogen metabolism, and sulfur metabolism; their loss may have contributed *C. glabrata*'s adaptation to the human gastrointestinal tract [28,41,42]. Interestingly, one classical example is again connected to nicotinic acid, paralleling *Shigella* spp.: *C. glabrata* has lost its ability for *de novo* nicotinamide adenine dinucleotide (NAD^+) biosynthesis and requires external nicotinic acid or niacin as precursors [43]. This auxotrophy allows it to detect the low niacin levels in the urinary tract and regulate, *via* lack of NAD^+-dependent histone de-acetylation, the expression of its virulence-associated Epa adhesins [44]. In connection to this, in a limited NAD environment, the epigenetic regulator Hts1 is inactivated and triggers derepression of genes involved in oxidative stress and fluconazole resistance, themselves major pathogenic traits of *C. glabrata* [45]. Thus, a gene loss enables the fungus to correctly detect the host environment and commence a virulence-associated genetic program.

Other clear examples are found in large and often redundant gene families of the environmentally acquired dimorphic pathogens of mammals, *Coccidioides* spp. and *Histoplasma capsulatum*. The first, along with the duplication of genes thought to be adaptive in the animal host [31], shows a reduction in genes encoding plant cell wall degrading enzymes, such as cellulases, cutinases, tannases, and pectinesterases, in stark contrast, e.g. to plant saprophyte *Aspergillus* species. *H. capsulatum* has experienced a similar reduction of plant matter-degrading enzymes during the evolution toward an animal pathogenic phenotype [31]. It requires no large leap of the imagination to assume the replication and expression of such genes to be detrimental in the human host, where they would lead to unnecessary energy expenditure and become potential immune-recognition targets within the host.

Antivirulence genes identified experimentally in host interactions

In laboratory evolution experiments, *C. glabrata* was shown to increase its virulence during long-term exposure to macrophages. During *in vitro* adaptation to the phagocytes, a single nucleotide mutation likely rendered a chitin synthase without function. The resultant change in growth morphology was associated with a transient increase in virulence and organ burden [46]. Moreover, in *C. neoformans*, the gene *ALL1*, involved in capsule formation, was shown to be downregulated during phenotypic switching to the so-called hypervirulent mucoid colony (MC) variant, which happens during chronical cryptococcal infections. This switching elicits damage-promoting inflammation and can lead to death of the host [47]. Jain et al. investigated the involvement of *ALL1* downregulation in the increased virulence of the MC variant. A knock-out mutant of *ALL1* was observed to lead to an ineffective immune response, failure to clear the pathogen, and decreased survival in animal models without any other impairment in fitness in the host environment [48]. Loss of *ALL1* influences capsule polysaccharides, which inhibit phagocytosis and impairs cell-mediated immune response. Furthermore, a H99L strain of *C. neoformans*, which was obtained by successive laboratory passages of the reference strain H99, shows inactivating mutations in the SAGA-associated factor gene *SGF29*. As a result, there is a reduction in histone acetylation and increased melanization. This was found to be associated with increased virulence in animal models and in addition, loss-of-function mutations in this gene were found in clinical isolates from patients with prevalent infections [49]. These examples show how spontaneous inactivation of certain genes can increase pathogen fitness and virulence during fungal infections.

In *C. albicans* and *C. glabrata*, cellular respiration affects host–pathogen interactions. In 2007, Cheng et al. [50] performed five serial passages of *C. albicans* through murine spleens by intravenous inoculation. They recovered a mutant with uncoupled oxidative phosphorylation, which was resistant to phagocytosis by neutrophils and macrophages. In long-term infections, this strain showed increased persistence and higher fungal burden in mice. In the case of *C. glabrata*, a clinical isolate that, as *petite* mutant, lacks fully functional mitochondria showed increased tissue burden in murine models compared to respiration-competent strains [51]. Thus, genes involved in respiration may be considered potential antivirulence genes in *Candida* species, which by inactivation can lead to increased fitness during infection.

Antivirulence genes identified through knock-out mutations

In 2016 Brown et al. [39] reviewed examples of single genetic mutations that cause hypervirulence, which are collected in the pathogen–host interaction database (www.PHI-base.org). Seventeen examples were found among fungal pathogens of humans and plants, and currently, more than 20 potential antivirulence genes have been identified in important opportunistic pathogens: *A. fumigatus, C. albicans, C. glabrata*, and *C. neoformans*. Many of these genes are involved in cell wall morphogenesis and responses to stress that are frequently connected to immune evasion and stress resistance. In 2005, Tsitsigiannis et al. described a triple-deletion mutant of *A. fumigatus* lacking the genes *ppoA, ppoB*, and *ppoC* [52]. These encode fatty acid oxygenases required for the biosynthesis of oleic and linoleic acid-derived oxylipins, which in turn coordinate sexual and asexual sporulation [53]. The mutant was hypervirulent in a murine model of invasive pulmonary aspergillosis and showed increased tolerance to H_2O_2 stress. The authors suggest an oxylipin-mediated cross talk that induces host defenses against the development of pulmonary and invasive aspergillosis. In *C. neoformans*, the regulator of G protein signaling, Crg1 is a key regulator of pheromone-responsive mating. A *CRG1* mutant shows largely increased virulence in the prevalent and clinically important MATα strains of the fungus: Mouse survival time after infection was shortened by 40%, and the lethal dose was 100-fold lower than that of wild-type strains. Here, the activation of mating due to the *CRG1* deletion may have caused the upregulation of the Ste12 pathway that promotes melanin formation. Melanin in turn can increase stress tolerance and fungal survival, or alter the host immune response, thereby increasing virulence [54]. In the yeast *C. albicans*, a mutant lacking the cell wall protein Pir32 was found to be hyperfilamentous and hypervirulent, with increased resistance to different stressors [55]. The authors suggest that the lack of Pir32 on the cell surface was compensated by an increased chitin content, which is known to promote antifungal resistance [56–58]. Thus, inactivation of genes with roles in cell wall biosynthesis in different species led to more adaptive and virulent phenotypes in experimental infections. Often, these seem to be mediated by compensatory stress responses of the fungal cell, which are induced by the lack of the gene in established networks. Such pre-stressed cells can be more resilient when facing the host immune response and therefore over-compensate the detrimental effect of the gene loss in the context of the host.

Other potential antivirulence genes have been identified counter-intuitively by knocking out genes which were expected to support virulence. For example, this is the case for the genes *tpsA* and *tpsB*, coding for trehalose-6-phosphate synthase of *A. fumigatus*. In *C. albicans* and *C. neoformans* [59–62], these genes mediate stress response and virulence, but in *A. fumigatus* their inactivation actually triggers cell wall alterations that lead to enhanced immune evasion and hypervirulence *in vivo* [63]. FleA is a lectin of *A. fumigatus* that binds fucosylated structures, which are abundant in the glycan coats of many plant and animal proteins. Kerr et al. [64] showed that FleA of *A. fumigatus* conidia binds to airway mucins which allow macrophages to effectively phagocytose them. Deletion of FleA accordingly reduces phagocytosis, and mice infected with *fleA*Δ conidia develop more severe pneumonia and invasive aspergillosis than with wild-type conidia. It is not certain why *A. fumigatus* and other pathogenic fungi have evolved to express such a protein, but it is thought that FleA can help to grow on carbohydrate-rich surfaces [64]. Therefore, the FleA lectin acts as an antivirulence factor during *A. fumigatus* infections, and its loss can be advantageous for *A. fumigatus* growing on human mucosae. This concept also explains why antivirulence factors are still present in pathogenic fungi of mammals. As most of them exist in the environment or as commensals most of the time, these genes are adaptive for the majority of possible niches. Only when they enter the host, the new selective forces can work to inactivate these genes. A closer look at clinical isolates from severely ill patients, in comparison with commensal or environmental strains would most likely reveal more potential antivirulence genes.

The concept and examples of effectors and avirulence genes in human pathogenic fungi

As described above, virulence factors contribute to the development of host damage and thus disease. However, as damage-associated factors, they can also trigger recognition by the host. This can happen either by a damage-mediated host response [18,19,25] or at an earlier stage due to antagonistic evolution (Figure 2): if the host has "learned" to recognize the virulence factor

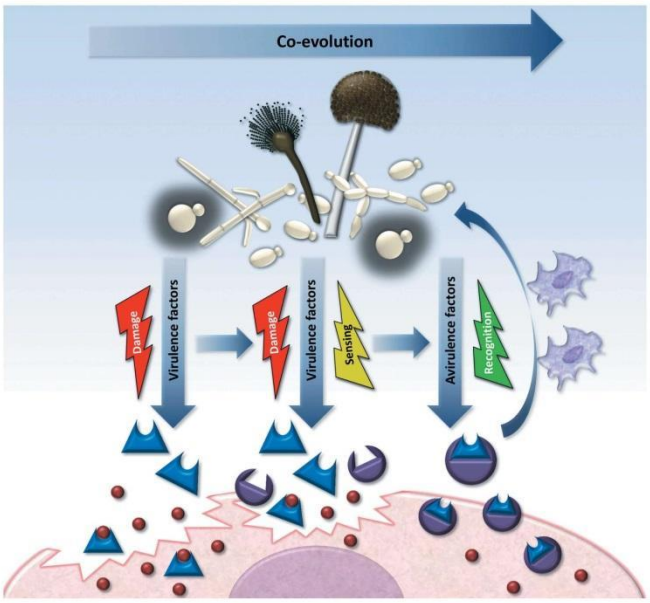

Figure 2. Evolution of a virulence factor to an avirulence factor as a result of antagonistic co-evolution. A virulence factor confers the pathogen with an adaptive advantage within the host environment, which allows the infection to progress. However, frequent host–pathogen interactions act as a selection pressure on the host side to develop a specific defense response. As a result of this co-evolution the host can develop receptors that specifically recognize the pathogen's virulence factor and trigger a specific (immune) response that counteracts and thereby abolishes the pathogen's virulence. Note that new avirulence factor can still serve as a virulence factor in susceptible hosts that have not yet developed the specific response.

and raise specific defense mechanisms against it even before damage commences. Common examples of such a coevolution are found in fungal pathogens of plants [20,21,65,66]. These fungi rely on effectors, which are generally secreted molecules that modulate the interaction between the fungus and the host at different steps of infection [20]. Not unlike the mammalian immune system, plants have developed a multi-layered defense against fungal pathogens. The initial, general response is triggered by microbe-associated molecular patterns and comprises unspecific antimicrobial compounds [20]. Plant-pathogenic fungi overcome this barrier with secreted effectors, which can suppress the host immune response, or manipulate host cell physiology to support fungal survival. For example, *Cladosporium fulvum* is a biotrophic fungal pathogen that causes leaf mold of tomato (*Solanum lycopersicum*). In the host, it releases the effector Ecp6 to sequesters chitin oligosaccharides detached from its own cell walls, and thereby prevents a triggering of the host immunity [67]. The corn smut agent, *Ustilago maydis*, secretes high amounts of the chorismate mutase Cmu1 during plant colonization. This enzyme reduces the levels of salicylic acid precursors and thus neutralizes salicylic acid-induced immune responses [68].

To counterattack the suppressing action of the fungal effectors on the first line of plant defense, plants have evolved a second layer of defense, termed effector-triggered immunity, through production of receptors that specifically recognize the pathogen virulence effectors. Thus, plant protein receptors detect the fungal effectors and trigger a hypersensitive response consisting on localized cell death to stop the propagation of the infection, rendering the plant resistant. Since this makes the fungi carriers of this specific effector avirulent in this specific plant species, these fungal effector-encoding genes are known as avirulence genes (*AVR*) (Figure 1). It is noteworthy that they contribute to either virulence or avirulence, depending on the present of a corresponding receptor gene (*R*) in plant host [20,69]. This host–pathogen interaction is known as gene-for-gene relationship, in which the host possesses a specific gene (*R*) whose product targets a certain virulence effector encoded by another specific gene of the pathogen (*AVR*) [70].

The effector Avr2 is present in *C. fulvum* and selectively binds and inhibits plant proteases involved in basal defense. In resistant plants, however, the receptor Cf-2 recognizes the antivirulence-protease and activates a hypersensitive response [71,72]. Such complex *R-AVR* interactions likely result from the long coevolution between plants and their pathogens: Effector evolution is therefore a trade-off between escaping detection and optimizing the virulence-related functions. In the long term, pathogen fitness may rely on the continuous emergence of novel effectors as replacement for those that are detected by the host. This implicates a strong evolutionary pressure on effectors, and genome-wide analyses of plant pathogenic fungi have in fact demonstrated a higher degree of positive selection in genes encoding secreted proteins compared with genes encoding non-secreted proteins [20,73–76].

With this in mind, can we expect to find similar mechanisms in human-fungal pathogens? Among the few species of pathogenic fungi of humans that live as a commensal within or in close contact with the host are *Candida* spp., for which we would expect the strongest signs of co-evolution [3]. The majority of human pathogenic fungi are opportunists that are adapted to environmental niches; however, effectors adapted to distinct niches may still confer adaptive advantages within the human body. This concept, where fungi gain pathogenic potential in environmental niches is known as "(environmental) virulence schools"[3]. It is thought that environmental fungi evolve to adhere to surfaces, form biofilms, compete with bacteria, acquire all necessary nutrients and deal with changes in temperature, pH, osmolarity and other physical stresses, all relevant factors for survival in the human host [10,12,77–79]. Moreover, it is known that *A. fumigatus, C. neoformans, Coccidioides* spp. and *H. capsulatum* are facing soil amoeba [3,15,80], which share many characteristics with human phagocytes [15]. Thus, the virulence attributes displayed by these fungi pathogens may be advantageous to defend both, against environmental phagocytes and phagocytes from animal hosts, and therefore, the study of the interaction of environmental fungi and amoeba may uncover potential virulence and avirulence genes.

Effectors of plant pathogens can act in the cytoplasm (when directly delivered *via* sophisticated systems – intracellular effectors) or the apoplast (the extracytoplasmic space – extracellular effectors) of plants to modulate the immune responses. Examples of analogous protein functions which act extracellularly can be found in both, commensal and environmental human pathogenic fungi. Here they are involved in host immune evasion or modulation in favor of the pathogen [14,81,82]. For example, the human complement system, an important contributor to innate immunity, is a common target for manipulation by fungal pathogens [83–85]. *C. albicans* and *A. fumigatus* express proteins that can bind or inactivate complement proteins: Secreted and cell surface-localized Pra1 [86–88],

Hgt1 [89], Gdp2 [90], and Gmp1 [91] of *C. albicans* can bind complement evasion-mediating human compounds like factor H, plasminogen, and others; the *C. albicans* Sap-family of aspartic proteases and the Alp1 protease of *Aspergillus* spp. can degrade the effector components of the terminal complement complex [92–94]. *Aspergillus* species also synthesize a soluble complement inhibitor, which prevents complement activation and opsonization [83]. Similarly, the secreted protein App1 of *C. neoformans* can inhibit complement receptor-mediated phagocytosis by macrophages [84,95]. *A. fumigatus* releases the metabolite gliotoxin which is immunosuppressive and able to induce apoptosis of monocytes and macrophages [96–99]. Lastly, *C. albicans* Sap proteases have been shown to have the potential to degrade host antibodies [100,101] and the two surface-bound member, Sap9 and Sap10, can inactivate antimicrobial peptides (AMP) released by the host [102]. Importantly, many of these proteins are themselves potent triggers of host responses: Sap proteases trigger inflammation [103] and Pra1 derives its name from being a pH-regulated antigen of *C. albicans* [104].

An example reminiscent of the gene-for-gene mechanism in plant pathogenesis is the recently discovered Melanin-sensing C-type Lectin receptor (MelLec) involved in immunity against *A. fumigatus* [105]. MelLec recognizes the naphthalene-diol unit of *A. fumigatus* melanin, one of the most important virulence factors of this fungus [106] . In mouse models and in humans this receptor seems to be required for resistance against *Aspergillus* infections [105]. The existence of a very specific receptor in the host for a fungal virulence factor thus renders the pathogen avirulent in otherwise healthy hosts.

Further examples of potential avirulence genes can be found in the most pathogenic species of the *Candida* group: *C. albicans*. This fungus possesses a hyphae-associated gene, *DUR31*, which not only contributes to epithelial damage but is also required in multiple stages of candidiasis, including surviving attack by human neutrophils and mediating endothelial damage [107]. It is thus required for full virulence *in vivo*. However, Dur31 also transports histatin 5 (Hst 5), a highly cytotoxic human AMP, into the fungal cell, thereby committing a suicide-like process [107]. Therefore, Dur31 is an indispensable protein for the normal progress of infection, but the host has "learned" to take advantage of it against the pathogen. This is not unlike the response of plants to certain fungal effectors (avirulence effectors) [108], and can be considered another potential example for a gene-for-gene relationship (in that case, histatin 5 exploiting the presence of Dur31). The heat shock protein Ssa1, present on the surface of hyphae, but not yeasts, of *C. albicans* is another example. It acts as an invasin, and a *ssa1*Δ/Δ mutant shows attenuated virulence in mouse models of both disseminated and oropharyngeal candidiasis [109]. However, Ssa1 also facilitates transport of the antimicrobial peptides Hst 5 and β-defensins, enabling their activity inside the fungal cell [110,111]. Furthermore, the fungal toxin candidalysin is considered an important virulence factor of *C. albicans* and one of the very few "classical virulence factors" of human pathogenic fungi [112,113], which directly damages host cells and allows fungal hyphae to cross the epithelial barriers [113,114]. However, candidalysin can also activate the epithelial "danger response" pathway [113,115]. This alerts the host to the presence of invasive, toxin-producing hyphae and induces protective immune responses [113]. In fact, it was shown that oral epithelial cells orchestrate innate Type 17 responses to *C. albicans* hyphae through candidalysin [116]. Thus, because of the dual property of this toxin as a damage agent and an activator of the immune system, it may be considered an avirulence factor in the immune competent host (and like its plant counterparts, a virulence factor in the susceptible host). Supporting this argument, two further recent studies have shown more dual, virulence and avirulence traits of candidalysin: on one hand, this fungal toxin contributes to killing of macrophages, but also activates the inflammasome, with production of the pro-inflammatory cytokine IL-1β and recruitment of other immune cells to the site of infection [117]. Similarly, it also promotes antifungal immunity in the central nervous system by activating the production of neutrophil-recruiting IL-1b and CXCL1 in microglia [118] . However, whether candidalysin acts as a virulence or an avirulence factor also depends on the site and tissue of infections: during vaginal infections, candidalysin triggers an immune response, which is associated with immunopathology and thus diseases [119]. Thus, virulence has not only to consider the pathogen and the host but also to consider the specificities of a particular host niche.

Conclusion

Is it helpful to argue in favor of applying the concept of antivirulence and avirulence genes to human pathogenic fungi, as we did in this review? We think yes, as the way we name and label biological phenomena determine to a great degree how we think of them. Not trying to fit everything in these traditional boxes, or maybe taking a different point of view and accepting new paradigms (or established ones from other fields) can thus help to ask new questions and bring science forward. For example, while the host-mediated, local destruction of tissue in

response to fungal growth is called a hypersensitive response in plants (and considered beneficial in limiting spread of the pathogen), it is seen as a damaging, overshooting immunopathology in mammals. In that manner, plant-pathogen effectors which elicit a hypersensitive response are treated as avirulence factors, while, e.g. candidalysin, when it causes localized immune-mediated, vaginal tissue damage [119], is seen as a virulence factor. In both cases, the fungal pathogens have the capacity for systemic infection of susceptible host, and the same immune mechanisms that stop their spread also lead to local tissue damage. This comparison admittedly glosses over many of the intricacies of the respective host–pathogen interactions, but it can be very helpful for researchers in fungal pathology on occasion to change their perspective and maybe the nomenclature they hold dear, in order to correctly ask the next important research question.

There are many possible benefits: The identification of antivirulence and avirulence genes in more pathogenic fungi would help us to comprehend the emergence and evolution of pathogenesis. As in bacterial pathogens, the reintroduction of functional antivirulence genes in their respective pathogens may allow to create vaccine candidates against fungal infections [120]. Moreover, search and identification of fungal effectors that trigger host immune responses would also uncover possible new virulence factors and potential antifungal targets. Thinking in terms of antivirulence and avirulence in the field of human-fungal pathogens will thereby, we hope, help to achieve a better understanding of fungal virulence and disease.

Acknowledgments

We thank the reviewers of this manuscript for their exceptional helpful comments.

SSL received funds from the German Graduate School of Excellence Jena School for Microbial Communication (JSMC). BH is supported by the Deutsche Forschungsgemeinschaft CRC/TR FungiNet Project C1, the Leibniz Association Campus InfectoOptics SAS-2015-HKI-LWC and the H2020–Marie Skłodowska-Curie Actions–European Training Networks grant agreements No 642095 - OPATHY and No. 812969 - FunHoMic.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft [SFB 124]; H2020 Marie Skłodowska-Curie Actions [812969]; H2020 Marie Skłodowska-Curie Actions [642095]; Leibniz-Gemeinschaft [SAS-2015-HKI-LWC].

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6.2 Manuscript II

Candida pathogens induce protective mitochondria-associated type I interferon signalling and a damage-driven response in vaginal epithelial cells

Marina Pekmezovic, Hrant Hovhannisyan, Mark S. Gresnigt, Elise Iracane, João Oliveira-Pacheco, **Sofía Siscar-Lewin**, Eric Seemann, Britta Qualmann, Till Kalkreuter, Sylvia Müller, Thomas Kamradt, Selene Mogavero, Sascha Brunke, Geraldine Butler, Toni Gabaldón, Bernhard Hube

Nature Microbiology. 2021. Published online 2021 Mar 22. DOI: 10.1038/s41564-021-00875-2

Summary:

The phylogenetically diverse species *Candida albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* are the main etiological agents of vulvovaginal candidiasis. This article shows the interaction between these species and vaginal epithelial cells during an *in vitro* model of infection. The four species exhibit highly species-specific transcriptional profiles, meaning that they display different pathogenicity patterns. In contrast, the host cells show a comparable response to all species at early stages of infections, which promotes a protective type I interferon response through a sub-lethal mitochondrial signaling. At later time points the host response shifts to a more species-dependent manner, as the species exert epithelial cell damage though species-specific mechanisms, such as secretion of the toxin candidalysin by *C. albicans.* This paper shows for the first time an epithelial response to *Candida* species in which an early commensal interaction is promoted, which at later time points diverges to a species-specific, damage-driven response.

The candidate is:
☐ First author ☐ Second author ☐ Corresponding author ☒ Coauthor

Estimated authors' contributions:

Author	Conception	Data analysis	Experimental	Writing	Provision of the material
MP	20%	20%	70%	15%	
HH	20%	30%		15%	
MSG	20%			10%	
EI		5%	5%	5%	
JP		5%	5%	5%	
SSL		5%	10%	5%	
ES		5%	5%	5%	
BQ		5%		5%	
TKal		5%	5%		
SMü		2.5%			
TKam		2.5%			
SMo		5%		5%	
SB		5%		5%	
GB		5%		5%	
TG	20%			10%	
BH	20%			10%	

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https://doi.org/10.1038/s41564-021-00875-2

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Candida pathogens induce protective mitochondria-associated type I interferon signalling and a damage-driven response in vaginal epithelial cells

Marina Pekmezovic[1,12], Hrant Hovhannisyan[2,3,10,11,12], Mark S. Gresnigt[4], Elise Iracane[5], João Oliveira-Pacheco[5], Sofía Siscar-Lewin[1], Eric Seemann[6], Britta Qualmann[6], Till Kalkreuter[1], Sylvia Müller[7], Thomas Kamradt[7], Selene Mogavero[1], Sascha Brunke[1], Geraldine Butler[5], Toni Gabaldón[2,3,8,10,11,13] ✉ and Bernhard Hube[1,9,13] ✉

Vaginal candidiasis is an extremely common disease predominantly caused by four phylogenetically diverse species: *Candida albicans*; *Candida glabrata*; *Candida parapsilosis*; and *Candida tropicalis*. Using a time course infection model of vaginal epithelial cells and dual RNA sequencing, we show that these species exhibit distinct pathogenicity patterns, which are defined by highly species-specific transcriptional profiles during infection of vaginal epithelial cells. In contrast, host cells exhibit a homogeneous response to all species at the early stages of infection, which is characterized by sublethal mitochondrial signalling inducing a protective type I interferon response. At the later stages, the transcriptional response of the host diverges in a species-dependent manner. This divergence is primarily driven by the extent of epithelial damage elicited by species-specific mechanisms, such as secretion of the toxin candidalysin by *C. albicans*. Our results uncover a dynamic, biphasic response of vaginal epithelial cells to *Candida* species, which is characterized by protective mitochondria-associated type I interferon signalling and a species-specific damage-driven response.

Vulvovaginal candidiasis (VVC) is among the most common fungal infections, affecting 70–75% of women at least once in their lifetime[1]. VVC is characterized by acute inflammation of the vaginal mucosa due to the overgrowth of normally commensal *Candida* species[2–4]. Although *Candida albicans* is the predominant cause of VVC, the prevalence of species like *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* has increased (reviewed in Makanjuola et al.[5]). Despite the shared genus name, these species are phylogenetically diverse and often have non-pathogenic close relatives, indicating that their ability to infect humans has emerged independently[6]. How these diverse *Candida* species interact with host cells has rarely been addressed on a comparative basis. Improved knowledge of similarities and species-specific characteristics of infection processes is crucial to understand the pathogenesis, improve diagnostics and therapy of candidiasis[7].

Research on host–fungi interactions has mainly focused on immune cells, which are considered crucial players in the defence against fungal infections[8,9]. However, epithelial cells play a fundamental role in shaping the host defence against fungi, which goes beyond their function as a physical barrier[10–15].

Studies in infection biology often focus on either the pathogen or host response, yet microbial pathogenesis can be best interpreted in the framework of dynamic host–microbe interactions. Dual RNA sequencing (RNA-seq) enables the combined assessment of the transcriptional responses of host and pathogen[16,17] and provides insights into the interactions of fungal pathogens with different host cells[17–23].

To elucidate general and species-specific interactions between vaginal epithelial cells and the four most prevalent VVC-causing *Candida* species, we applied dual RNA-seq and an in vitro infection model. Our experimental design allows pathogens to deploy their arsenal of pathogenicity factors without restriction by the immune system. Furthermore, it facilitates specific investigation of epithelial recognition and defence mechanisms, which constitute the first line of defence against infecting fungi.

Our results reveal that fungal transcriptomes show species-specific patterns during infection, probably reflecting the independently evolved pathogenic potential of *Candida* species. Vaginal epithelial cells display a biphasic response: an early protective type I interferon (IFN) response, which is mediated by sublethal

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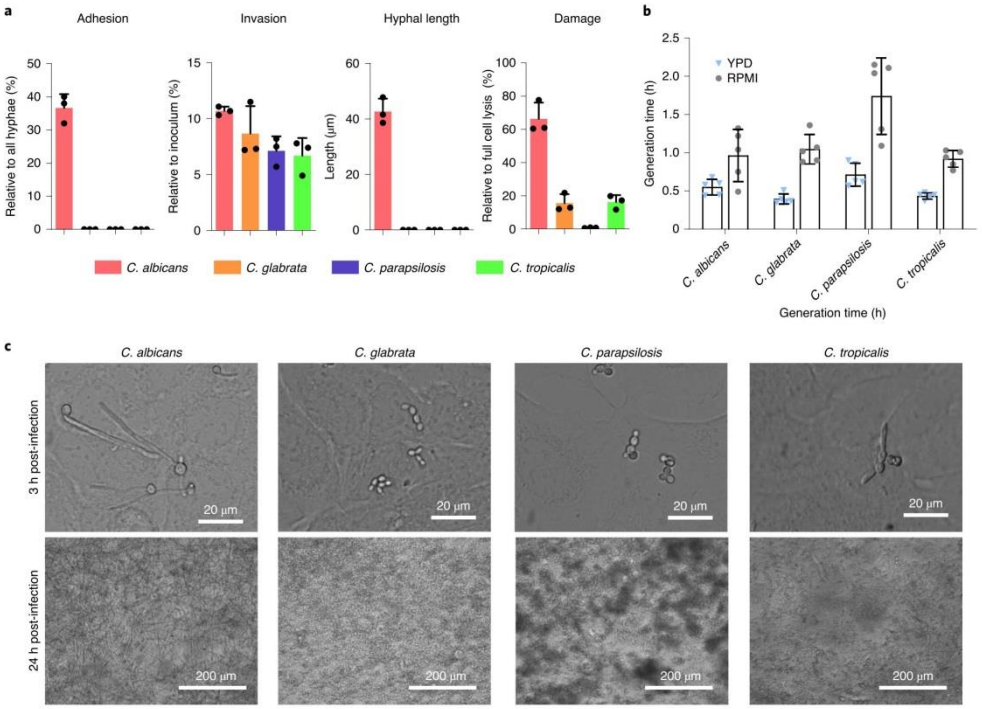


Fig. 1 | Pathogenicity patterns of four *Candida* species in the in vitro vaginal epithelial infection model. a, Adhesion, determined as the percentage of *Candida* cells from the original inoculum that adhered to vaginal epithelial cells at 1 h post-infection; invasion, determined as the percentage of *Candida* cells that invaded the vaginal epithelial cells at 3 h post-infection; hyphal length (μm) recorded at 3 h post-infection; necrotic damage, measured by the quantification of LDH activity in the supernatant and presented as a percentage respective to total lysis (maximum damage control) at 24 h post-infection. All values are presented as mean ± s.d. of $n = 3$ independent in vitro infection experiments. **b**, Generation times of *Candida* species in YPD or RPMI 1640 medium (used for vaginal epithelial cell infections) measured in 24-h growth curves. All values are presented as the mean ± s.d. of $n = 5$ independent experiments. No statistically significant difference in growth between species in neither YPD nor RPMI was observed (one-way ANOVA with Greenhouse–Geisser correction and Tukey's multiple comparisons test). **c**, Micrographs of *Candida* morphology at 3 h post-infection and confluent biofilms at 24 h post-infection on vaginal epithelial cells. Micrographs are representative of $n = 3$ independent experiments with similar results.

mitochondrial signalling; and a damage-associated late response that depends on species-specific pathogenicity mechanisms.

Results

***Candida* species-specific pathogenicity patterns.** To study the interaction of the four most common *Candida* species causing VVC with vaginal epithelial cells, we first assessed their adhesion, invasion and damage potential as well as their growth rates and morphologies (Fig. 1a). Despite the similar adhesion rates of all species, only *C. albicans* switched to hyphal growth, invaded epithelial cells and induced necrotic cell damage. Non-invading *C. glabrata* and *C. tropicalis* cells caused low damage levels. *C. glabrata* grew only in the yeast morphology, whereas occasional pseudohyphae were observed for *C. tropicalis*. Finally, *C. parapsilosis* remained in the yeast morphology during the entire course of infection, forming cell aggregates, but did not invade or caused damage. All species exhibited similar growth rates (Fig. 1b) and showed a biofilm layer 24 h post-infection, thus excluding the possibility that differences in epithelial damage resulted from different proliferation rates (Fig. 1c). These results show species-specific pathogenicity patterns, involving different morphologies, levels of invasion and damaging capacity.

Species-specific transcriptional responses to epithelial cells. Subsequently, we investigated whether these differential pathogenicity patterns were reflected in the transcriptional responses of both fungal and epithelial cells (see the experimental set-up in Extended Data Fig. 1 and cross-mapping analysis between human and yeast sequencing reads in Supplementary Files 1–4). The transcriptional dynamics of each *Candida* species throughout the infection were analysed (Fig. 2). All species induced rapid transcriptional responses following infection with increasing numbers of differentially expressed genes over the course of infection (Fig. 2a and Supplementary Files 5–8). Gene Ontology (GO) enrichment analysis revealed the function of differentially expressed genes (Fig. 2b and Supplementary File 9). We observed species-specific functional enrichments, albeit with some commonalities. At 3 h post-infection, *C. albicans* and *C. glabrata* activated carbohydrate catabolic

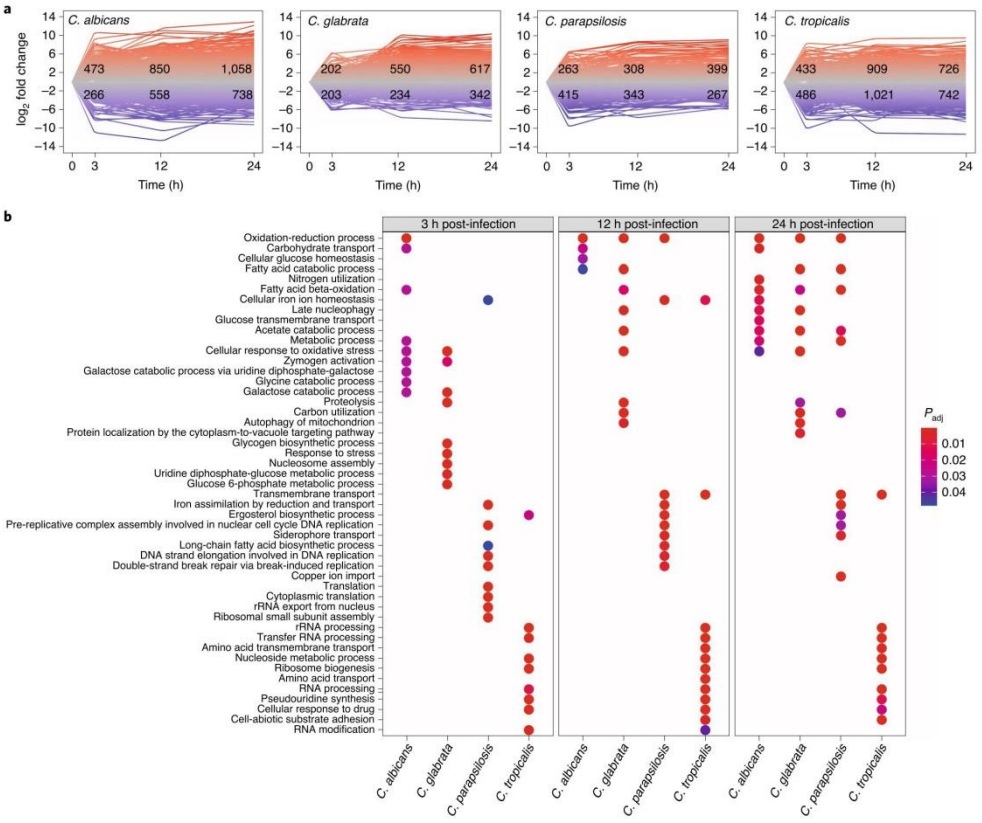


Fig. 2 | Dynamics of transcriptomic changes of the four *Candida* species investigated in this study at different time points. a, *Candida* species transcriptome dynamics plots from RNA-seq data based on $\log_2$(fold change) of expression during the course of infection compared to *Candida* adapted to RPMI culture medium (time point 0). Each line corresponds to the relative fold change of expression levels of a single gene. The numbers on the plots indicate a number of differentially expressed genes ($\log_2$(fold change) >1.5, $P_{adj} < 0.01$, upregulated (red), downregulated (blue)). **b**, GO term enrichment analysis of upregulated genes (category 'biological process') in the four *Candida* species. The *x* axis indicates the fungal species, the *y* axis indicates GO terms. Only significant ($P_{adj} < 0.05$) GO enrichments are shown. Differentially expressed gene analyses were done using DESeq2 v.1.26.0; comparisons against time point 0 were done using the two-sided Wald test. GO enrichment analysis was done using clusterProfiler v.3.14.3, which was used to perform the hypergeometric test. Adjustments of *P* values for differentially expressed gene and GO enrichment were done by Benjamini–Hochberg procedure. See Supplementary File 9 for the full list of enriched GO terms for each species, time point, up- or downregulation and GO category.

processes and stress response pathways. *C. parapsilosis* upregulated, among others, genes related to iron transport, ribosome assembly and translation. In contrast, *C. tropicalis* differentially expressed genes were mainly related to RNA processing, ribosome biogenesis and ergosterol biosynthetic processes. At later stages, similar functional enrichments were observed across the species. The GO terms oxidation-reduction process, fatty acid beta-oxidation, iron homeostasis and acetate catabolism were enriched in at least three species throughout the infection.

When comparing differentially expressed genes across species, a remarkably distinct pattern was observed for each pathogen (Extended Data Fig. 2). Analysis of the distribution of species-specific (without orthologues in the other species), partially shared (with orthologues in one or two of the other species) and fully shared (1-to-1 orthologous genes in all species) differentially expressed genes (Extended Data Fig. 2a) revealed that species-specific and partially shared genes constitute a substantial proportion (31–72%). Moreover, species-specific genes are more likely to be differentially expressed than fully shared genes (chi-squared test $P < 0.05$, except for *C. tropicalis*). Even orthologous genes present in all four species showed species-specific differential expression (Extended Data Fig. 2b). Consistently, principal component analysis (PCA) based

on orthologous gene expression showed species-specific clusters (Extended Data Fig. 2c).

Gene coexpression analysis was used as an independent approach to investigate commonalities and differences of fungal transcriptional responses. By constructing host–pathogen interaction coexpression networks, highly interconnected gene clusters (modules) were defined and their biological functions were inferred by GO term analysis. In each fungal infection scenario, we detected numerous modules of coexpressed genes (22–28 modules; Supplementary File 10).

Based on fully shared genes, we then assessed whether the fungal genes in the coexpressed modules were conserved across species. Distinct modules were observed for each *Candida* species with few shared genes. On average, only 5% of orthologous genes were shared between any modules of different species (Extended Data Fig. 3). Therefore, the genes in the coexpression modules functionally showed a large species specificity (Supplementary File 10) with few exceptions. The modules with the highest similarity, that is, module3 in *C. glabrata* and module3 in *C. tropicalis*, were both enriched for genes associated with DNA replication. Interestingly, we observed modules related to adhesion in *C. albicans* (module11) and *C. tropicalis* (module13), respectively, possibly related to shared virulence features of these two species.

Infection-specific differentially expressed genes of *Candida* species. Comparisons of *C. albicans* gene expression during infection of oral epithelium or vascular endothelium, and growth in the tissue culture medium, revealed that only a fraction of genes were specifically expressed during interactions with host cells[20]. This indicates that most of the genes induced during interaction with the host are also required for growth in culture media.

To investigate whether such a phenomenon also occurs during interaction with vaginal cells, controls of fungal cells grown in culture medium only were investigated (Extended Data Fig. 1 and Supplementary Table 1). A subtraction of the differentially expressed genes in medium from those expressed on epithelial cells revealed a large overlap between the differentially expressed genes during infection and in culture medium (Fig. 4 and Supplementary Files 5–8). Infection-specific genes are mostly species-specific (Extended Data Fig. 4) and GO enrichment analysis identified different functional enrichments depending on the species (Supplementary File 9).

These were characterized by genes involved in mitochondrial electron transport and ATP synthesis for *C. glabrata*, ergosterol biosynthesis, sulphite and manganese ion transport for *C. parapsilosis* and ribosome biogenesis and ribosomal RNA processing for *C. tropicalis*. No functional enrichments were identified in the upregulated, infection-specific *C. albicans* genes, yet the downregulated genes showed enrichment in three GO terms related to white-opaque phenotypic switching. These downregulated genes include *WOR1*, a master regulator inducing the less virulent opaque state[24].

In summary, distinct transcriptional patterns for each yeast species during infection were observed, suggesting highly species-specific strategies to cope with epithelial cells.

Epithelial transcriptomic responses to *Candida* species. To shed light on how the *Candida* species-specific pathogenicity and transcriptional patterns influence the host response, epithelial transcriptome responses to infections were analysed (Fig. 3a,b). The epithelial cell transcriptome dynamics showed a bias towards upregulation of genes at the initial stages of infection (Fig. 3a), which is consistent with previous findings[25].

When compared against the total number of differentially expressed genes (Fig. 3c, top), the proportion of common differentially expressed genes induced by infection with any of the four species decreased throughout the time course—8.8% of common differentially expressed genes at 3 h post-infection, and 7.6 and 6.4% at 12 h and 24 h post-infection, respectively. A similar pattern is observed when comparing common differentially expressed genes to the differentially expressed genes induced specifically by each fungal species (Fig. 3c, bottom). The larger fraction of shared differentially expressed genes at the early time points suggests that the response to the different yeast species is more conserved at the early infection stages while increased species specificity is observed at the later stages (Fig. 3b,c).

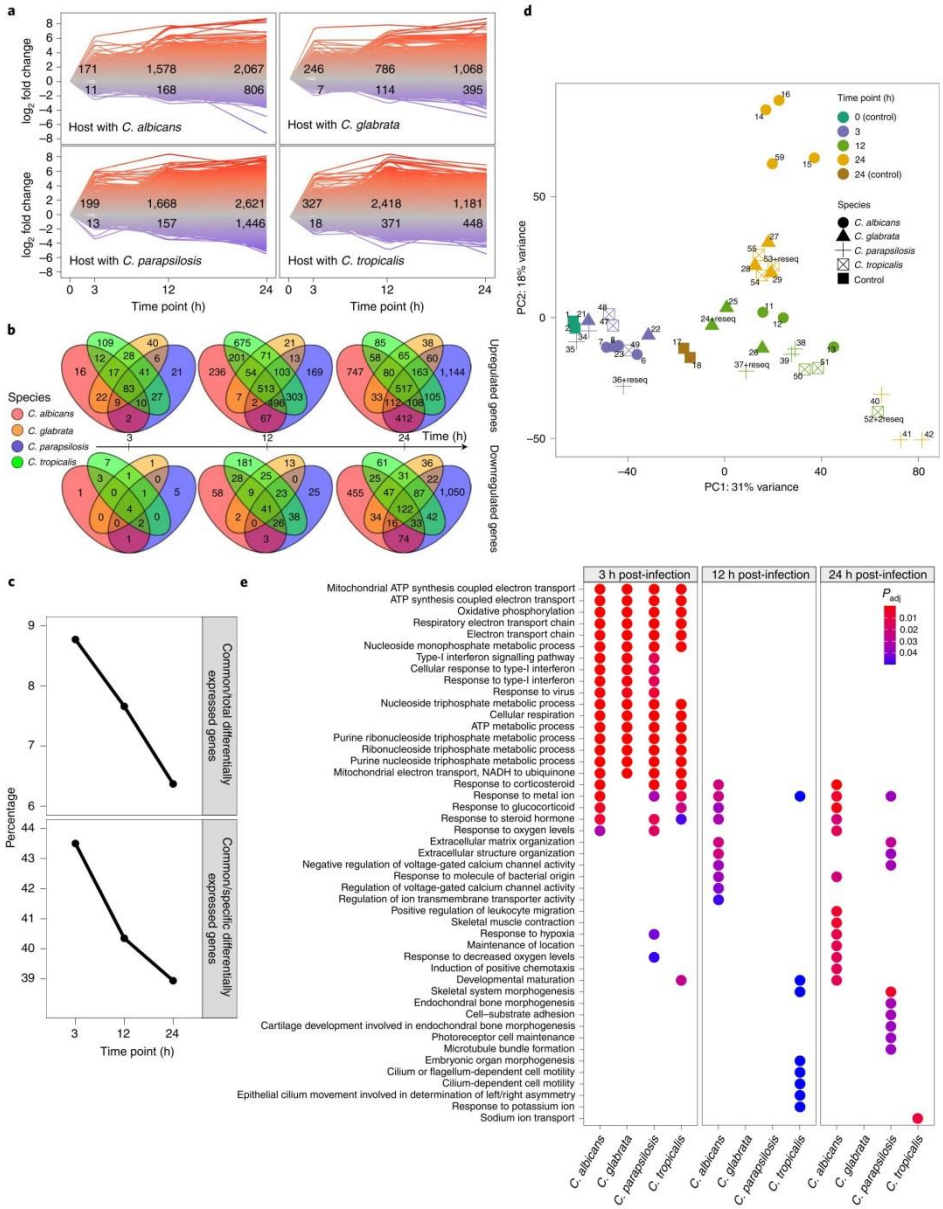
PCA analysis of the gene expression of epithelial cells revealed a similar pattern (Fig. 3d). The tight clustering at 3 h post-infection indicates that epithelial cells exhibit a uniform transcriptional response to the four *Candida* species at the early stages of infection, in contrast to the fungal transcriptional profiles. However, the epithelial transcriptomes diverge from 12 h post-infection onwards; at 24 h post-infection we observed three distinct clusters of responses to the different species. The transcriptional response of epithelial cells to *C. tropicalis* and *C. glabrata* showed high similarity, being different from the responses to *C. albicans* or *C. parapsilosis*. Functional GO term enrichment analysis revealed a similar trend: at the early time point, GO terms associated with mitochondrial processes are enriched for all species and of type I interferon (IFN) responses for all species except *C. tropicalis*. At the later stages, species-specific terms appeared (Fig. 3e).

Based on these results, we decided to unravel the basis of the two observed key phenomena: (1) the uniform early transcriptional response related to mitochondria and type I IFN signalling; and (2) the divergence of the host transcriptome response at the later stages of infection.

Uniform early responses to *Candida* infections. The upregulation of genes associated with the respiratory electron transport chain (Fig. 3e) in epithelial cells at the early time points with all *Candida* species included induction of all mitochondrial genes (Fig. 4a). The response was dependent on viable fungi being in direct contact with the epithelial cells (RNA-seq, Extended Data Fig. 6 and Supplementary Files 5–8; quantitative PCR with reverse

Fig. 3 | Transcriptome dynamics of vaginal epithelial cells on exposure to four *Candida* species. a, Transcriptome dynamics plots from RNA-seq data based on $\log_2$(fold change) compared to time point 0. Each line corresponds to the relative fold change of expression levels of a single gene. The numbers on the plots indicate the number of differentially expressed genes ($\log_2$(fold change) >1.5, $P_{adj} < 0.01$, upregulated (red), downregulated (blue)). **b**, Venn diagrams showing similarities and differences of human differentially expressed genes in response to *Candida* species. **c**, Proportion between shared differentially expressed genes and the total number of differentially expressed genes (top) and proportion between shared differentially expressed genes and the differentially expressed genes induced exclusively by each fungal species (bottom). **d**, PCA biplot of all analysed human samples. The labels of the data points correspond to sample identifiers, where 'reseq' indicates that the sample was sequenced more than once (Supplementary Table 1 for further details). **e**, GO term enrichment analysis for upregulated genes (category 'biological process') of the host at different time points. The *x* axis indicates the infecting *Candida* species. Only significant ($P_{adj} < 0.05$) GO enrichments are shown. Differentially expressed gene analyses were done using DESeq2 v.1.26.0; comparisons against time point 0 were done using a two-sided Wald test. GO enrichment analysis was done using clusterProfiler v.3.14.3, which was used to perform the hypergeometric test. Adjustments of *P* values for differentially expressed gene and GO enrichment were done by Benjamini–Hochberg procedure.

transcription (RT–qPCR), Fig. 4a). Similarly, our coexpression network analysis identified host modules with functional enrichment of mitochondrial genes and oxidative phosphorylation across all infections (Supplementary File 10). These observations suggest that mitochondria-associated processes are triggered in epithelial cells on infection with *Candida* species.



Host mitochondria have recently been identified as hubs of the innate immune responses[26,27]. In particular, mitochondrial signalling is known to activate type I IFN signalling pathways[28]. We observed enrichment of GO terms (Fig. 3e) and upregulation of interferon-stimulated genes (ISGs) associated with the type I IFN response[29] on exposure of epithelial cells to the four *Candida* species (Fig. 4a). Since type I IFN responses are implicated in antiviral host defence, additional metagenomic analyses were performed and no viral contamination was detected.

The connection between ISG expression and mitochondrial functions of the host was characterized during early *Candida* infection. Morphological changes of the mitochondrial network in epithelial cells were observed, changing from reticular (uninfected) to fragmented (infected), and an accumulation of mitochondria around the nucleus (Fig. 4b). Some mitochondria in infected epithelial cells lost their integrity and changed their shape but not in uninfected cells (Fig. 4c,d). Additionally, endoplasmic reticulum regions surrounded these altered mitochondria, suggesting mitophagy of damaged mitochondria. Interestingly, mitochondria were also localized frequently around the invading hyphae of *C. albicans* (Fig. 4c).

Mitochondrial membrane potential ($\Delta\Psi$m), a key indicator of mitochondrial health, indicated depolarization in epithelial cells infected with any of the four species (Fig. 4e). This change in $\Delta\Psi$m is associated with the production of mitochondrial reactive oxygen species (mtROS), which are critical players in the regulation of immune signalling pathways[30]. Epithelial mtROS levels were increased on infection with all four species (Fig. 4f). Finally, release of mitochondrial DNA (mtDNA) into the cytosol was observed during infection with all *Candida* species (Fig. 4g). The release of mtROS and mtDNA was not detected with killed *C. albicans* cells or when contact was restricted using a transwell system (Fig. 4e,f). This supports our notion that viable *Candida* cells in direct contact with epithelial cells induce mitochondrial dysfunctions at both transcriptional and biochemical levels (Fig. 4h).

During infection with bacteria or viruses, host mitochondria can release mtDNA into the cytosol acting as a damage-associated molecular pattern (DAMP) that activates immune pathways[31]. Cytosolic mtDNA can bind the DNA sensor cyclic GMP-AMP synthase and promote stimulator of interferon genes (STING)-IRF3-dependent signalling to induce a type I IFN response[28]. In line with this, depletion of epithelial mtDNA (Fig. 5a) prevented upregulation of ISGs after *Candida* infections (Fig. 5b). In addition, transfection of uninfected epithelial cells with amplified mtDNA induced ISG expression (Fig. 5c). Transfection of uninfected epithelial cells with total DNA from epithelial cells only induced ISGs when the transfected DNA contained mtDNA (Fig. 5d), which supports the role of mtDNA in the induction of ISG expression (Fig. 5e).

Although mitochondrial dysfunction is a hallmark of cellular apoptosis, no apoptotic or necrotic epithelial cells were observed during the early stages of infections (Extended Data Fig. 5). This was expected since mitochondrial dysfunctions were only transiently observed. Later during infection, we observed necrotic cell death but no increase in apoptosis compared to the uninfected control (Extended Data Fig. 5). The A-431 cell line lacks functional p53, an important apoptosis inducer[32]. However, we observed similar mitochondrial depolarization in primary vaginal cells on *Candida* infections, while apoptosis levels did not differ between infected and uninfected cells (Extended Data Fig. 5). Additionally, treatment of A-431 cells with the apoptosis inducer staurosporine excluded that the observed mitochondrial signalling and induction of ISG expression were related to apoptosis (Extended Data Fig. 5).

The function of many ISGs is poorly characterized but induction is associated with protection against viral infections[33]. To gain insights into their role during *Candida* infection, selected ISGs (*IFI6*, *MX2*, *CMPK2*) were silenced in epithelial cells before infection with *C. albicans*. *IFI6* was selected since it was previously observed to be induced by *C. albicans*[34], *MX2* was among the most highly upregulated common genes (this study) and *CMPK2* encodes a protein with mitochondrial localization[35]. The level of epithelial damage was increased once these ISGs were silenced (Fig. 6a). While stimulation of cells with IFN-β (0.1 ng ml^{-1}) before infection resulted in reduced damage (Fig. 6b); blocking IFN-α/β receptor (IFNAR) signalling led to increased damage (Fig. 6b). These data illustrate that type I IFN signalling increases epithelial resistance to *Candida* infection.

Neutrophil recruitment and activation is a hallmark of vaginal candidiasis[36]. Pro-inflammatory mediators, such as IL-6 or IL-1β, which are characteristic for these events, were not produced during *Candida* infection (Fig. 6c), while IL-1α and IL-8 levels increased (Fig. 6d), which correlates with the level of damage. IL-8 levels also increased on IFNAR blocking (Fig. 6d), suggesting a relationship between epithelial type I IFN signalling and the initiation of pro-inflammatory responses, which can drive immunopathology in VVC.

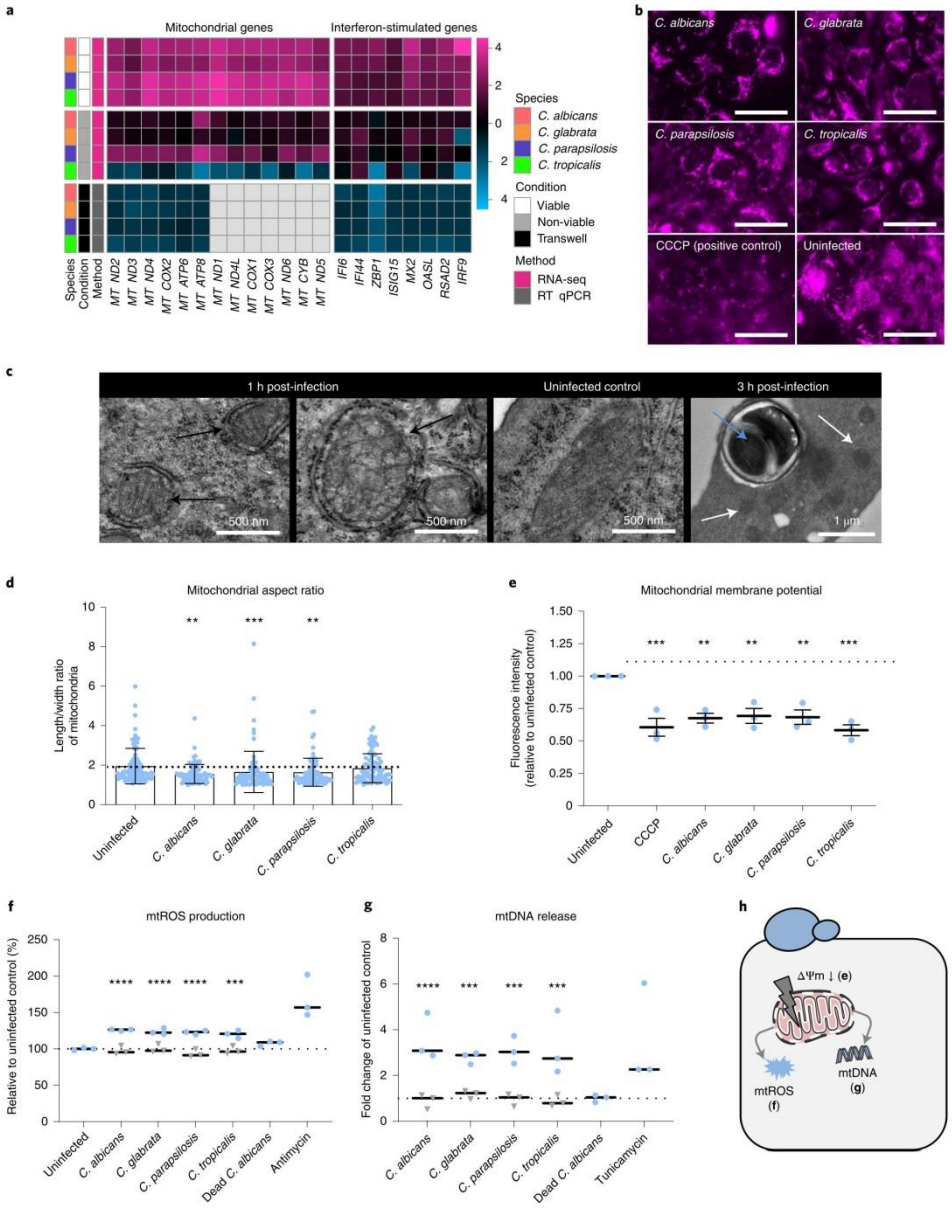
When exposing neutrophils to the culture supernatants of epithelial cells infected with *Candida* species, IL-8 release was observed (Fig. 6e). IL-8 levels further increased when the IFNAR receptor was blocked in the same setting (Fig. 6e). This suggests that type I IFN signalling in epithelial cells restricts pro-inflammatory responses and subsequent neutrophil activation (Fig. 6f).

Damage-driven transcriptional responses. While the initial phases of infection showed a conserved epithelial response, this response separated into different trajectories at later stages. Considering that host cell damage is a major determinant of pathogenicity[37–39], we hypothesized that *Candida* species-specific differences in the damaging potential (Fig. 1 and Extended

Fig. 4 | *Candida* species induce mitochondrial responses in vaginal epithelial cells. a, Expression of mitochondrial and interferon-stimulated genes with all four species in direct contact with epithelial cells, with non-viable *Candida* cells, and in the transwell system. Data for infection in direct contact and with non-viable *Candida* cells were derived from RNA-seq data ($\log_2$(fold change) > 1.5 for *C. albicans*, *C. glabrata* and *C. parapsilosis*; $\log_2$(fold change) > 1.3 for infections with *C. tropicalis*), while transwell experiments were done additionally as $n = 3$ independent experiments; transcription levels were analysed using RT–qPCR. **b**, Mitochondrial imaging by fluorescence microscopy using MitoTracker Deep Red FM at 1 h post-infection. Scale bars, 50 µm. **c**, TEM analysis of mitochondria in uninfected and *C. albicans*-infected epithelial cells (1 and 3 h post-infection): loss of mitochondrial integrity in infected epithelial cells (white arrows); mitochondria (black arrow) localized around the invading hyphae of *C. albicans* (blue arrow) at 3 h post-infection. **d**, Mitochondrial aspect ratio quantified by TEM at 1 h post-infection ($n \geq 80$ mitochondria examined over 1 independent experiment). **e**, Mitochondrial membrane potential change at 1 h post-infection (positive control 100 µM of CCCP). **f**, Levels of mtROS production at 1 h post-infection (positive control 100 µM of antimycin). **g**, Levels of mtDNA released into the cytosol (qPCR) on infection with *Candida* species at 6 h post-infection (positive control 10 µM of tunicamycin). **h**, Schematic model of the events associated with mitochondrial dysfunctions resulting in mtROS production and mtDNA release. All data are derived from $n = 3$ independent experiments, unless indicated otherwise (**d**). Representative microscopy images (**b**–**c**) were taken from $n = 3$ biological replicates and similar results were observed. All values are presented as the mean ± s.d. relative to the uninfected (−) control (dotted lines on (**d**–**g**)). Statistical significance is indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ (Kruskal–Wallis test with two-sided Dunn's multiple comparison (**d**) and one-way ANOVA with Dunnett's multiple comparisons test (**e**–**g**). Credit: graphics in **h** adapted from Servier under a Creative Commons licence CC BY 3.0.

Data Fig. 6), reflected by the pattern observed in the PCA plot (Fig. 3d), drive the different transcriptional responses during late infection.

Epithelial cell damage during *C. albicans* infection is mediated by the cytolytic toxin candidalysin[40,41]. Deletion of the *ECE1* gene, which encodes candidalysin, renders *C. albicans* almost unable to



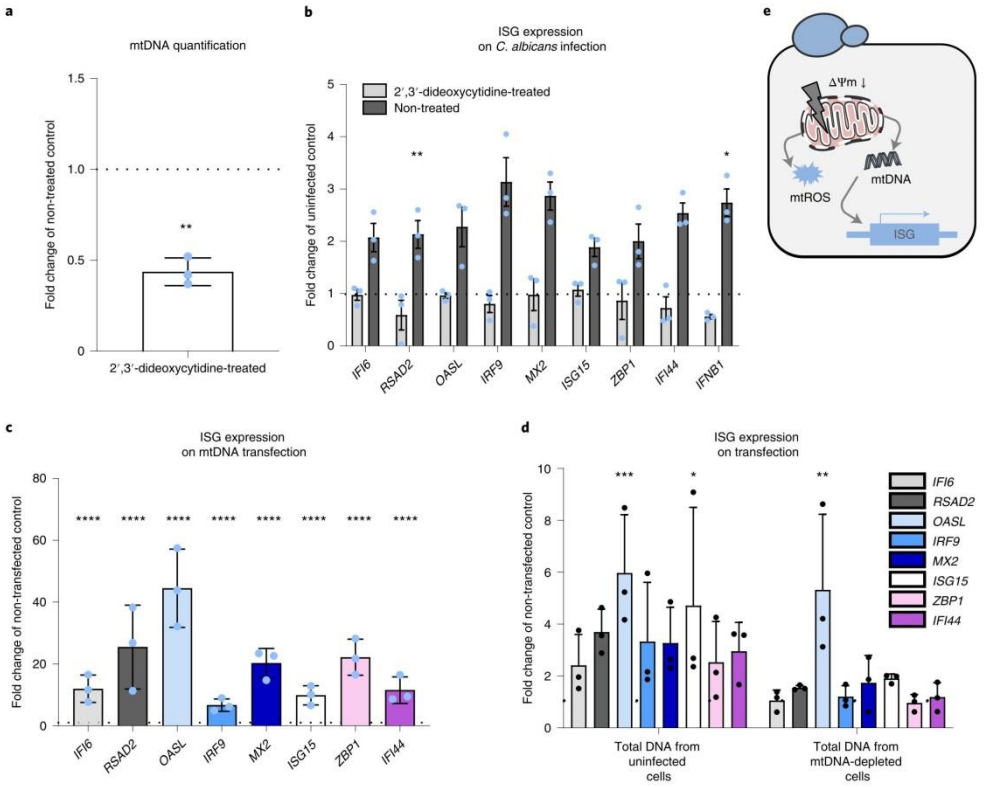


Fig. 5 | Role of mtDNA in the induction of type I IFN in vaginal epithelial cells. a, Depletion of mtDNA level by treatment of vaginal epithelial cells with 2′,3′-dideoxycytidine for 6 d, measured by qPCR. **b**, Relative expression (RT–qPCR) of selected ISGs (*IFNB1, IFI6, RSAD2, OASL, IRF9, MX2, ISG15, ZBP1* and *IFI44*) in *C. albicans*-infected, mtDNA-depleted epithelial cells at 3 h post-infection. **c**, Relative expression (RT–qPCR) of selected ISGs on transfection of epithelial cells with amplified mtDNA fragments at 6 h post-transfection. **d**, Relative expression of selected ISGs (RT–qPCR) in epithelial cells transfected with total DNA obtained from vaginal epithelial cells with and without mtDNA depletion at 6 h post-transfection. **e**, Schematic model of ISG expression induction by released mtDNA. All values are presented as the mean ± s.d. relative to the uninfected/non-transfected (−) control (dotted lines) of $n = 3$ independent experiments. Statistical significance is indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$ (two-tailed one-sample *t*-test (**a**), two-way ANOVA and Sidak's multiple comparisons test (**b**,**d**) and one-way ANOVA with Dunnett's multiple comparisons test (**c**)). Credit: graphics in **e** adapted from Servier under a Creative Commons licence CC BY 3.0.

inflict damage to epithelial cells, despite normal growth, adhesion, filamentation and invasion properties[25,40].

To determine whether candidalysin-driven epithelial cell damage might dictate the transcriptional response of epithelial cells, the transcriptional response on interaction with the *C. albicans ece1*Δ/Δ mutant was investigated. The epithelial transcriptional response to the candidalysin-deficient mutant was notably similar to the response to the non-damaging species *C. parapsilosis* (Extended Data Figs. 6 and 7). This confirms a pivotal role for host cell damage as the major driver of epithelial transcriptional responses to *Candida* infections. GO term enrichment analysis of 774 genes, specifically upregulated on exposure to damaging wild-type *C. albicans*, showed no significant enrichment for any process. However, previous studies demonstrated that candidalysin induces c-Fos and mitogen-activated protein kinase-driven release of the pro-inflammatory cytokines IL-1α and IL-1β and the chemokine IL-8 in vaginal epithelial cells[12,25]. Manual inspection revealed similar responses including upregulation of *HBEGF*, *CXCL1*, *CXCL2*, *IL1A*, *IL1B*, *CXCL8* and *CSF2* and genes associated with the 'danger' response pathway *FOS*, *JUN* and *DUSP1* (refs. [12,25]). This confirms that epithelial damage and pro-inflammatory signals that drive neutrophil recruitment induced by *C. albicans* depend almost exclusively on candidalysin.

Discussion

In this study, we dissected the interaction of the main four *Candida* species that cause VVC with human vaginal epithelial cells. Large-scale dual transcriptomic analysis of human and fungal cells during the course of infection revealed common and species-specific *Candida* pathogenicity patterns. We observed a

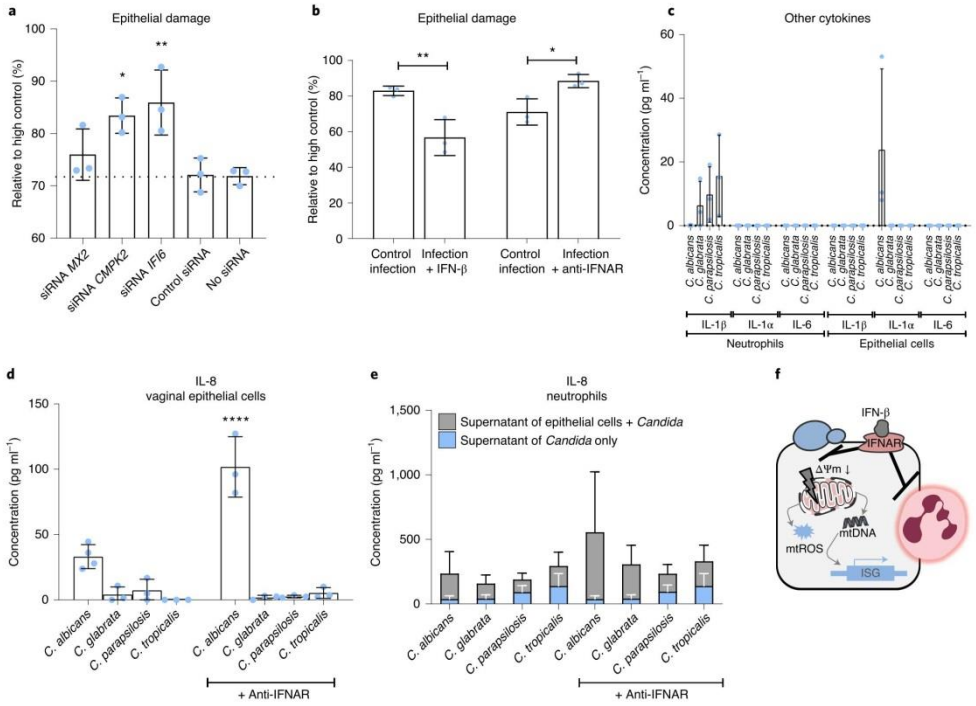


Fig. 6 | Type-I IFN signalling increases epithelial resistance and suppresses innate immune activation. a, Epithelial damage caused by *C. albicans* 24 h post-infection after RNA interference for selected ISGs (*MX2, CMPK2, IFI6*). Transfection with control siRNA, consisting of a scrambled sequence that will not lead to the specific degradation of any mRNA, was used as a control (dotted line). **b**, Epithelial damage caused by *C. albicans* 24 h post-infection without and with 0.1 ng ml^{-1} of IFN-β and the addition of anti-IFNAR antibody. **c**, Levels of IL-6, IL-1β and IL-1α secretion by neutrophils incubated with supernatants from infected epithelial cells and by epithelial cells infected with *Candida* species 24 h post-infection. **d**, Levels of IL-8 secretion by infected epithelial cells 24 h post-infection with or without the addition of anti-IFNAR antibody. **e**, Levels of IL-8 secretion by neutrophils after 24-h incubation with supernatants from epithelial cell infections with or without the addition of an anti-IFNAR antibody. Control supernatants of *Candida* cells alone were included to ensure that the neutrophils responded to secretions of epithelial cells rather than the fungus (blue bars). **f**, Schematic model of the proposed role of the type I IFN pathway in immune regulation and protection against *Candida* infection. All values are presented as the mean ± s.d. of $n = 3$ independent experiments (except $n = 4$ for IL-8 production by epithelial cells on *C. albicans* infection). Statistical significance is indicated as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{****}P \leq 0.0001$ (one-way ANOVA with Dunnett's multiple comparisons test (**a**) or Tukey's multiple comparisons test (**b**,**d**)). Credit: graphics in **f** adapted from Servier under a Creative Commons licence CC BY 3.0.

biphasic host response to *Candida* species, which is characterized by an early common mitochondria-induced type I IFN signalling and diverged responses at later stages depending on the species-specific capacities to inflict damage to the vaginal epithelial cells.

It has previously been hypothesized that phylogenetically diverse *Candida* species independently acquired their ability to colonize and infect humans and thus are expected to use distinct sets of pathogenicity mechanisms[6,23]. Our study empirically supports this hypothesis by showing that the main VVC pathogens express species-specific transcriptional responses and pathogenicity patterns on contact with vaginal epithelial cells, even when only orthologous genes were considered.

Similarly, the epithelial transcriptional responses at the late stages of infection were specific depending on the *Candida* species. These diverse patterns paralleled the varying damaging capacities of the four *Candida* species. We confirmed fungal-induced damage as the major driver of epithelial responses by infecting epithelial cells with the non-damaging candidalysin-deficient *C. albicans ece1*Δ/Δ mutant. The epithelial transcriptional response to this mutant did not resemble the response to wild-type *C. albicans*, but instead was similar to the response to the non-damaging species *C. parapsilosis* at the late stages of infection. This finding confirms the crucial role of candidalysin during interaction of *C. albicans* with vaginal epithelial cells leading to DAMP release that can catalyse immunopathology during vaginal infections[25].

In contrast, the epithelial response towards the different *Candida* species was highly uniform at the early stages. This initial response was driven by common epithelial processes rather than by convergent activities (such as virulence programmes) of the tested *Candida* species. For example, independent of their viability, *DOCK8* expression was upregulated after infection with any of the four *Candida* species (Supplementary Files 5–8). Although *DOCK8* has multiple

signalling functions, it was suggested that it promoted immune responses to diverse external stimuli[42]. Several studies associated *DOCK8* with mucocutaneous candidiasis due to impaired T_H17 differentiation[43–45]. Thus, it is tempting to speculate that *DOCK8* may regulate the recognition of *Candida* species by epithelial cells.

Mitochondria-encoded genes and genes associated with the type I IFN response pathway were uniformly upregulated. While type I IFN responses are associated with viral infections[46], type I IFN responses have been observed in peripheral blood mononuclear cells infected with *C. albicans*[34]. Additionally, type I IFN responses were recently shown to dysregulate host iron homeostasis and enhance *C. glabrata* infection[47]; the type I IFN-inducing RIG-I helicase MDA5 has been associated with systemic and chronic mucocutaneous candidiasis[48]. Finally, IFNαR1 signalling is crucial for efficient host defence against systemic candidiasis in mice[49].

Our data show that type I IFN signalling, induced by vaginal epithelial cells in response to *Candida* species, increases epithelial resistance to infection and dampens pro-inflammatory responses.

Such an immune response may be relevant in host niches colonized by commensal microbes that need to be tolerated without induction of inflammation. For example, intestinal epithelial cells regulate the durability and specificity of immune responses and guide the immune system to differentiate between commensal and pathogenic microbiota via expression of type I IFN and ISGs[50–52]. Since *Candida* species are commensals of the vaginal mucosa[53], the epithelial type I IFN pathway may maintain the threshold between commensalism and pathogenicity and regulate antifungal immunity. Our results show a protective role for ISGs and IFNAR signalling in increasing epithelial resistance to *Candida*-induced damage while reducing potentially detrimental pro-inflammatory responses. Supporting this, Li et al.[54] showed that administration of human IFNα-2b decreased the inflammation and vaginal epithelial damage in a rat VVC model. The combined effects, immunomodulation and epithelial antifungal resistance, may be crucial to restrict *Candida* species to commensalism and avoid inflammation-driven pathology. Moreover, this highlights the type I IFN response as a potential target for host-directed therapy aimed at improving epithelial resistance and preventing immunopathology. Such a therapy could benefit VVC patients that fail to recover with antifungal treatment alone, a phenomenon often observed in women with recurrent VVC[55].

At the early infection stage, genes encoded by mtDNA, in particular genes coding for the respiratory electron transport chain, were upregulated. Apart from the well-established roles in metabolism and energy production, mitochondria are central hubs in innate immunity[26,27]. Mitochondrial dysfunction, resulting in mtROS and mtDNA release into the cytosol, can act as a DAMP and activate various signalling pathways[26–28,56–58], including induction of cytokine production[59] and type I IFN responses[60]. Intriguingly, altered mitochondrial function at the early stages of infection were observed in different host cell types on infections with various bacterial pathogens[61–64], including *Chlamydia trachomatis*[65], *Chlamydia pneumoniae*[66], *Listeria monocytogenes*[67] and the parasite *Toxoplasma gondii*[68]. However, these mechanisms have not yet been observed during fungal infections. We observed that mitochondria in *Candida*-infected vaginal epithelial cells changed shape and lost integrity, had decreased membrane potential and released mtROS and mtDNA. The release of mtDNA was observed to act as a DAMP that activates the type I IFN pathway during *Candida* infections of the vaginal epithelium. This activation may potentially occur through the STING pathway, as shown previously for *Streptococcus pneumoniae*[69], at the level of post-translational modifications[70].

To maintain epithelial integrity and mount an effective epithelial host defence while preventing detrimental inflammatory responses, such a mitochondrial response must occur on a sublethal level[59]. Accordingly, no changes in apoptosis were observed over the course of infection. The non-lethal mitochondrial dysfunction was also independent of necrosis, which was only observed at the later stages. Likewise, we observed consistent activation of the type I IFN pathway, which is suppressed by apoptotic caspases[71]. Consequently, induction of apoptosis abrogated expression of ISGs. Similar studies with *L. monocytogenes* and *T. gondii* showed that mitochondrial dysfunction was uncoupled from the apoptotic pathway[67,68]. During infection of epithelial cells with diverse microbes, the mitochondrial apoptosis apparatus can be activated at a low level, which is insufficient to induce apoptosis[59,72]. This phenomenon has been termed limited mitochondrial outer membrane permeabilization (MOMP), or 'minority MOMP', and induces pro-inflammatory cytokine production via STING.

Viruses, bacteria and parasites can induce minority MOMP, thereby contributing to cytokine release during infection[59]. We propose that this mechanism plays a significant role in epithelial sensing of *Candida* species, induction of epithelial antifungal immunity and modulation of immune responses via type I IFN signalling.

It remains to be determined how *Candida* species initiate the mitochondria-induced epithelial type I IFN response. We observed mitochondrial signalling at stages when *C. albicans* had not yet invaded or damaged epithelial cells, whereas all other *Candida* species failed to invade epithelial cells. Therefore, we propose that the induction of mitochondrial signalling may rely on sensing of pathogen-associated molecular patterns (PAMPs).

In summary, we identified species-specific pathogenicity patterns of *Candida* species infecting vaginal epithelial cells, which are reflected at the transcriptional level during the course of infection. In contrast, vaginal epithelial cells exhibit a conserved response at early stages, but a diverse, damage-driven response at later stages. The conserved response was characterized by non-lethal mitochondrial signalling, which induced a type I IFN response that protects against *Candida*-induced damage and modulates pro-inflammatory responses. This acts as a common pathway of host–pathogen interactions between vaginal epithelial cells and *Candida* pathogens.

Methods

Fungal strains and culture conditions. *C. albicans* SC5314 (ref. [73]), *Candida glabrata* ATCC 2001 (obtained from ATCC), *C. tropicalis* DSM 4959 (obtained from the German Collection of Microorganisms and Cell Cultures), *C. parapsilosis* 73-037 (ref. [74]) and *C. albicans ece1*Δ/Δ[40] were used in this study. For all experiments, single colonies were picked from yeast extract peptone dextrose (YPD) agar plates and grown overnight in liquid YPD medium in an orbital shaker at 180 r.p.m. at 30 °C (*C. albicans*, *C. tropicalis* and *C. parapsilosis*) or 37 °C (*C. glabrata*). Yeast cells were then collected by centrifugation (20,000*g*, 1 min), washed twice with PBS and adjusted to 2×10^6 yeast cells per ml^{-1}.

In vitro vaginal epithelial infection model. To mimic the vaginal epithelium, A-431 epithelial cells (ACC 91) were used. These cells are derived from a vulva epidermoid carcinoma and routinely used to model the vaginal mucosa[75,76]. A-431 cells were authenticated using short tandem repeat analysis (DNA fingerprinting) and routinely tested for the absence of *Mycoplasma* contamination. Epithelial cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Bio&SELL) in a humidified incubator at 37 °C and 5% CO_2. For infection, epithelial cells were seeded in 6-well plates (3×10^5 cells per well) and cultured for 2 d. On the day of infection, the medium in each well was replaced with 1.5 ml of RPMI 1640 without FCS and incubated for 30 min to allow cells to adjust to the change of medium. In subsequent bioinformatics analyses, we considered the control samples at 30 min in this medium as the 0 h time point. Epithelial cells were subsequently infected with *Candida* cells (1.5 ml of 2×10^6 yeast ml^{-1} in RPMI 1640 without FCS) and incubated at 37 °C and 5% CO_2. Samples for RNA isolation were collected at different time points: 3, 12 and 24 h post-infection. More specifically, the well content was removed and replaced with 500 µl of RLT buffer (QIAGEN), containing 1% β-mercaptoethanol (Roth). Cells were detached using a cell scraper (<3 min), immediately shock-frozen in liquid nitrogen and stored at −80 °C until further use (see 'RNA isolation and pooling'). As controls, *Candida* cells alone and epithelial cells alone were incubated for 30 min (0 h control: C0) and 24 h (24 h control: C24) and samples for RNA isolation were collected as described above.

RNA isolation and pooling. Collected samples were defrosted on ice and centrifuged for 10 min (20,000*g*, 4 °C). The supernatant was transferred to a

new microcentrifuge tube and used to isolate human RNA (RNeasy Mini Kit; QIAGEN), according to the manufacturer's instructions. Fungal RNA was isolated from the pellet using a freezing–thawing method, as described previously[77]. Both human and fungal RNA concentrations were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies). To subsequently achieve sufficient sequencing depth of both counterparts for a robust differential gene expression analysis[78], corresponding fungal and human RNA samples were pooled in a 2:3 ratio by weight for further library preparation and sequencing. Before pooling, we first checked whether this strategy would result in ambiguous read mappings between the host and pathogen data after sequencing and data analysis. To assess the rates of cross-mapping, that is, reads originated from human but mapped to fungi and vice versa, which can bias expression level quantifications, we used CROSSMAPPER v.1.1.0 (ref. [79]), which simulates reads from multiple reference genomes/transcriptomes, maps the data back to the concatenated reference sequences and reports the rates of cross-mapping. We used the 'RNA' mode of CROSSMAPPER and simulated and back-mapped 20 and 40 million 2×50 and 2×75 reads for each fungal species and human, respectively. In all cases, the pooling and sequencing strategy resulted in virtually no cross-mapping between human and yeast data (Supplementary Files 1–4).

Growth curves. *Candida* cells were adjusted to 10^6 yeast ml^{-1} either in YPD or RPMI medium. Growth was monitored in 96-well-plates by measuring the absorbance at 600 nm every 30 min for 24 h at 37 °C in a microplate reader (Tecan M-Plex). Before each measurement, plates underwent 10-s orbital shaking followed by 10-s waiting time. The OD_{600} values were converted into log_2 and the generation time was calculated from the slope of the exponential growth phase. The experiment was repeated five times.

Ultraviolet killing of *Candida*. *Candida* cells from overnight cultures were collected by centrifugation, washed twice with PBS and adjusted to approximately 5×10^7 yeast ml^{-1} in PBS. The suspension was transferred to a Petri dish as a thin liquid layer (10 ml) and exposed to 4 doses of 100–120 mJ per cm^2 in an ultraviolet (UV) CROSSLINKER (CL 508S; Uvitec). The efficiency of UV killing was evaluated by plating 50 µl of the sample onto YPD agar and incubated for 48 h at 30 °C.

Adhesion assay. Epithelial cells were infected with *Candida* yeast cells as described above and incubated for 1 h. Non-adherent *Candida* cells were removed by rinsing with PBS. Subsequently, epithelial cells with adhered *Candida* were fixed with Roti Histofix 4% (Roth). Adherent *Candida* cells were stained with Alexa Fluor 647 conjugate of succinylated concanavalin A (Invitrogen) and visualized using a fluorescence microscope (Leica DM5500B; Leica DFC360 FX). Pictures of each sample were taken until a total of 100 adherent cells were counted. Adhesion was calculated based on the average of *Candida* cells counted in each picture with a defined area. This number was expressed as a percentage of adhered cells versus inoculated cells[57].

Invasion assay and hyphal length. Epithelial cells were infected with *Candida* cells as described above and incubated for 3 h. Non-adherent *Candida* cells were removed by rinsing with PBS and samples were fixed with Roti Histofix 4%. Extracellular, non-invasive fungal components were stained by concanavalin A. After rinsing with PBS, epithelial cells were permeabilized in 0.5% Triton X-100 for 10 min. Next, fungal cells were stained with Calcofluor white (Sigma-Aldrich) and visualized by fluorescence microscopy. The total hyphal length was noted as well as the percentage of invasive hyphae (only Calcofluor white-stained), counted from at least 100 hyphae per strain for each biological replicate.

Epithelial damage assay. Epithelial cells were infected with *Candida* cells as described above and incubated for 24 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured as a marker for necrotic epithelial damage[80] using a Cytotoxicity Detection Kit (Roche) according to the manufacturer's instructions. The background LDH value of uninfected epithelial cells (low control) was subtracted and the corrected LDH release was expressed as the percentage of high (full lysis) control (maximum LDH release induced by the addition of 0.25% Triton X-100 to uninfected epithelial cells for 5 min) unless otherwise stated. For the protection effect experiments, 0.1 ng ml^{-1} of IFN-β (InvivoGen) or neutralizing anti-human IFNAR2 antibody (4 ng ml^{-1}; PBL InterferonSource) were added to epithelial cells 3 h before infection.

Transwell assay. Epithelial cells were seeded in 24-well plates (1×10^5 cells per well) in RPMI 1640 with FCS and incubated for 2 d at 37 °C and 5% CO_2. After medium exchange with 750 µl of RPMI 1640 without FCS, transwell inserts (polycarbonate membrane inserts with 0.4 µm pore size; Corning), loaded with 250 µl of *Candida* suspension (4×10^6 yeast ml^{-1}), were placed in the wells. After 3 h of incubation, the inserts were discarded and human RNA samples were collected and isolated as described above.

RT–qPCR. Isolated RNA (500 ng) was treated with DNase I (Fermentas) according to the manufacturer's recommendations and subsequently transcribed into complementary DNA using 0.5 µg of $Oligo(dT)_{12-18}$ Primer, 200 U of Superscript III Reverse Transcriptase and 40 U of RNaseOUT Recombinant RNase Inhibitor (Thermo Fischer Scientific). The cDNA obtained was diluted 1:5 and used for qPCR with the GoTaq qPCR Master Mix (Promega Corporation) in a CFX96 thermocycler (Bio-Rad Laboratories). The expression levels were normalized against β-actin or 18S ribosomal RNA. All primers used are listed in Supplementary Table 2.

Measurement of epithelial cell mtDNA release. The release of mtDNA in response to infection was measured using the protocol of Bronner and O'Riordan[81] with some modifications. Briefly, epithelial cells were seeded in 6-well plates and infected as described above. After 6 h of infection, the medium and non-adherent *Candida* cells were removed. After the addition of 200 µl of the cell membrane detergent IGEPAL CA-630 (1%, NP-40; Sigma-Aldrich), cells were loosened by scraping. Lysates were incubated on ice for 15 min and centrifuged (12,000*g*, 15 min, 4 °C). The supernatant was used to isolate human mtDNA from the cytosolic fraction using the DNeasy Blood & Tissue Kit (QIAGEN), according to the manufacturer's instructions. Finally, cytosolic human mtDNA was measured by qPCR using 18S rRNA as a reference[81]. Results are presented relative to an uninfected control. Tunicamycin (10 µM; Sigma-Aldrich) was used as a positive control, as an endoplasmic reticulum stress inducer that leads to mitochondrial dysfunction[82]. The same procedure was carried out on yeast cells only to exclude that fungal cells would also release mtDNA following this protocol. The lysis step did not cause any lysis of yeast cells and no DNA was detected after the isolation procedure, confirming that the DNA obtained from the infected epithelial cells originated from epithelial cells only.

Measurement of $\Delta\Psi$m. The $\Delta\Psi$m was assessed using the dye MitoTracker Deep Red FM (Thermo Fisher Scientific, excitation (Ex)/emission (Em) = 644/665 nm). Epithelial cells were seeded and infected in 6-well plates and stained with 20 nM of MitoTracker Deep Red FM for 15 min at 37 °C. Epithelial cells were then detached using Accutase and fixed with Roti Histofix 4%. Fuorescence was quantified by flow cytometry (BD FACSVerse: BD Biosciences), counting 10,000 events; the data were collected using the BD FACSuite v1.0.6.5230 software and analysed with FlowJo v.10.2. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a protonophore that causes mitochondrial depolarization, was used as a positive control (100 µM).

Epithelial cell mtROS detection. Production of mtROS was measured using a Mitochondrial Reactive Oxygen Species Detection Assay Kit (Cayman Chemical). Epithelial cells were seeded and infected in a black clear-bottom 96-well plate; measurement of mtROS production of infected and uninfected cells was done at 1 h post-infection according to the manufacturer's instructions. Antimycin A (100 µM), which induces superoxide radicals leakage from mitochondria, was used as a positive control.

RNA interference assay. The RNA interference assay was used to silence the expression of selected ISGs (*IFI6*, *MX2* and *CMPK2*). Small interfering RNA, control siRNA (siRNA-A; catalogue no. sc-37007), siRNA transfection reagent and siRNA transfection medium were purchased from Santa Cruz Biotechnology. Epithelial cells were seeded in a 6-well plate and transfected with 1 µg of siRNA according to the manufacturer's instructions. After 48 h, cells were infected with *C. albicans*; 3 h post-infection, RNA was isolated and silencing of selected genes was confirmed using RT–qPCR. LDH release was measured 24 h post-infection.

mtDNA depletion assay. Epithelial cells were seeded in 6-well plates and incubated for 2 d. Once confluent, 200 µM of 2′,3′-dideoxycytidine (Jena Bioscience) was added to the medium and epithelial cells were incubated for 6 d. mtDNA depletion was confirmed by quantifying mtDNA using qPCR, as described above.

Transfection of epithelial cells. MtDNA was PCR-amplified from the entire human mitochondrial genome in 17 overlapping fragments as described previously[83]. Epithelial cells were transfected with amplified mtDNA fragments (Fig. 5c) or total DNA isolated from epithelial cells with and without their mtDNA depleted (Fig. 5d). Total DNA was isolated using the DNeasy Blood & Tissue Kit. A total of 2 µg ml^{-1} of DNA was used to transfect epithelial cells using the UltraCruz Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. After 6 h, RNA samples were collected and the expression of ISGs was quantified by RT–qPCR.

Apoptosis/necrosis assay. Epithelial cells were seeded in black 96-well plates and infected with *Candida* cells as described above. At 3 and 24 h post-infection, epithelial cells were stained for apoptotic (Apopxin Green Indicator, Ex/Em = 490/525 nm), necrotic (7-aminoactinomycin D, Ex/Em = 546/647 nm) and healthy cells (CytoCalcein Violet 450, Ex/Em = 405/450 nm) using the Apoptosis/Necrosis Assay Kit (Abcam). Fluorescence was measured in a microplate reader (Tecan M-Plex). Staurosporine (1.2 µM; Sigma-Aldrich) was used as a positive control, while uninfected epithelial cells were used as a negative control.

Apoptosis induction. Epithelial cells were seeded in 96-well plates and infected with *Candida* as described above with the addition of staurosporine (1.2 µM) simultaneously with infection. After 6 h, RNA samples were collected and the expression of ISGs was quantified by RT–qPCR. Results were compared to infected cells incubated in the medium without staurosporine.

Collection of epithelial cell supernatants. Epithelial cells were infected with *Candida* cells as described above and incubated for 24 h in the presence or absence of neutralizing anti-human IFNAR2 antibody. Supernatants were collected and stored at −80 °C until use (see Methods sections 'Cytokine release' and 'Neutrophil cytokine production'). The supernatants of only *Candida* cells grown in the absence of epithelial cells were included as control.

Cytokine release. Epithelial cells were infected with *Candida* cells as described above and incubated for 24 h. The release of IL-6, IL-8, IL-1α and IL-1β was measured with commercially available human enzyme-linked immunosorbent assay kits (IL-6, IL-8, IL-1β, Invitrogen; IL-1α, R&D Systems) according to the manufacturers' instructions.

Blood donors. Human peripheral blood was collected from healthy volunteers ($n=3$) with ethical approval and after obtaining written informed consent. This study was conducted according to the principles expressed in the Declaration of Helsinki version 2008. The blood donation protocol and use of blood for this study were approved by the institutional ethics committee of Jena University Hospital (permission no. 2207–01/08).

Neutrophil cytokine production. Primary human neutrophils were isolated from blood using a previously published protocol[84] and seeded in a 24-well plate (5×10^5 cells ml^{-1}). Neutrophils were exposed for 24 h to the supernatants of epithelial cells that had been infected with each of the *Candida* species (24 h post-infection) to determine whether pro-inflammatory mediators released by epithelial cells played a role in neutrophil stimulation. The control supernatants of *Candida* cells alone were included to ensure that the neutrophils responded to the secretions of epithelial cells rather than the fungus (blue bars on Fig. 6e). After incubation, cytokine release was measured using an enzyme-linked immunosorbent assay as described above.

Fluorescence microscopy. Epithelial cells were seeded in µ-Slide 8 Well (IBIDI) and infected with *Candida* cells as described above. At 1 h post-infection, epithelial cells were stained with 100 nM of MitoTracker Deep Red FM for 15 min at 37 °C and washed and fixed with Roti Histofix 4%. Fluorescence imaging was done with the Cell Observer microscope (Carl Zeiss) with fluorescence settings at 644 and 665 nm. CCCP was used as a positive control (100 µM). Image acquisition was done in a fully blinded manner to avoid potential bias.

Transmission electron microscopy and imaging. Cells were fixed by adding glutaraldehyde (2.5% (v/v) final) to the growth medium. After 1 h, the cell layer was gently scraped off the surface, collected as a pellet by centrifuging at 600*g* and washed three times with PBS. After fixation in osmium tetroxide (1% (w/v) in distilled water) for 1 h, dehydration in ascending ethanol series with post-staining in uranyl acetate was performed. Afterwards, samples were embedded in epoxy resin (Araldite) and sectioned ultrathin (60 nm) using an ultramicrotome (Leica Ultracut E; Leica Biosystems). After mounting on filmed copper grids and post-staining with lead citrate, the sections were studied in a transmission electron microscope (EM 902 A; ZEISS) at 80 kV. Images were acquired with a 1k FastScan CCD camera (TVIPS). The mitochondrial aspect ratio (the ratio of length/width) was measured using ImageJ version 1.43h by analysing at least 80 mitochondria for each condition[85] in 1 biological replicate. Irregular structures were excluded from analysis. All transmission electron microscopy (TEM) analyses were conducted in a fully blinded manner to avoid potential bias in image acquisition and analyses.

Primary vaginal cells. Primary human vaginal epithelial cells (catalogue no. PCS-480-010) were obtained from ATCC and cultured in vaginal epithelial cell basal medium (catalogue no. PCS-480-030; ATCC), supplemented with components from the Vaginal Epithelial Cell Growth Kit (catalogue no. PCS-480-040; ATCC). Cells were not authenticated but they were routinely checked for the absence of *Mycoplasma* contamination. Apoptosis/necrosis and mitochondrial membrane potential assays were performed as described for A-431 cells.

RNA-seq library preparation and sequencing. Library preparation for RNA-seq was performed with the TruSeq Stranded mRNA Sample Prep Kit v2 (catalogue no. RS-122-2101/2; Illumina) according to the manufacturer's instructions unless otherwise stated. One microgram of total RNA was used for poly(A)-mRNA selection using streptavidin-coated magnetic beads. Samples were then fragmented to approximately 300 base pairs (bp); subsequently, cDNA was synthesized using reverse transcriptase (SuperScript II; Invitrogen) and random primers. The second strand of the cDNA incorporated deoxyuridine triphosphate in place of deoxythymidine triphosphate. Double-stranded DNA was further used for library preparation. It was subjected to A-tailing and ligation of the barcoded TruSeq adaptors. All purification steps were done using AMPure XP beads (Agencourt). Library amplification was performed by PCR on size-selected fragments using the primer cocktail supplied in the kit. Final libraries were analysed using the Agilent DNA 1000 chip (Agilent) to estimate the quantity and check fragment size distribution; they were then quantified by qPCR using the KAPA Library Quantification Kit (Kapa Biosystems) before amplification with Illumina's cBot. To avoid potential batch effects, all samples were randomly distributed on the sequencing flow cells.

Libraries were sequenced with 2×50 ($n=21$), 2×75 ($n=70$) and 2×150 ($n=1$) read lengths on the Illumina HiSeq 2500 system (2×50 bp) and HiSeq 3000 (the rest) at the Genomics Unit of the Centre for Genomic Regulation, Barcelona, Spain. Samples that contained mixed fungal and human RNA were sequenced for (on average) almost equal to 65 million reads (Supplementary Table 1 and Extended Data Fig. 1) to achieve sufficient sequencing depth for robust downstream analysis[78].

Bioinformatics data analysis. FastQC v.0.11.6 (ref. [86]) and MultiQC v.1.0 (ref. [87]) were used to perform the quality control of raw sequencing data. Read trimming, when necessary, was performed by Trimmomatic v.0.36 (ref. [88]) with TruSeq3 adaptors using the 2:30:10 parameters and discarding reads shorter than the sequenced read length.

For read mapping and quantification, we used the splice junction-sensitive read mapper STAR v.2.5.2b[89] using the basic two-pass mode and default parameters. For samples comprising either fungal or human RNA, reads were mapped to the corresponding reference genomes. In the case of pooled samples containing RNA from both host and pathogen, data were mapped to concatenated human and corresponding yeast reference genomes. For human data, we used the primary genome assembly GRCh38 and genome annotations from the Ensembl database release 89 (last accessed on 8 August 2017 (ref. [90])). Reference genomes and genome annotations for *C. albicans* SC5314 (assembly 22), *C. glabrata* CBS138 and *C. parapsilosis* CDC317 were obtained from the *Candida* Genome Database (CGD, last accessed on 17 August 2017 (ref. [91])). From the phased reference genome and annotations of *C. albicans*, we selected haplotype A to perform further analysis to avoid substantial rates of ambiguously mapped reads. The reference sequence and annotations for *C. tropicalis* were obtained from the RefSeq database (last accessed on 9 August 2017 (ref. [92])). The genes missing from the RefSeq genome annotations were manually added from the *Candida* Gene Order Browser[93]. GFF genome annotation files were converted to GTF format using the gffread utility v.0.9.8 (ref. [94]). We used Centrifuge v.1.0.4 (ref. [95]) to test the presence of viral contamination in our dataset, by remapping the reads that mapped either to the human or fungal reference genomes to the whole National Center for Biotechnology Information nucleotide database (downloaded on 23 of March 2018). No traces of contamination were observed.

Differential gene expression analysis was performed using the Bioconductor package DESeq2 v.1.26.0 (ref. [96]) using the read counts obtained with STAR mapping. For human samples and each fungal species, we compared time point 0 with the other time points throughout the course of infection by Wald test using the contrast option of DESeq2. To detect any statistically significant changes of expression throughout the course of infection, we also used a likelihood ratio test of DESeq2, by dropping the 'time' component of the formula design. Genes with a $\log_2$(fold change) > 1.5 and adjusted P (P_{adj}) < 0.01 were considered differentially expressed unless otherwise stated. To account for possible batch effects in the experiments involving *C. albicans ece1*Δ/Δ and non-viable fungal cells, we applied the RUVg function of the Bioconductor package RUVseq v.1.20 (ref. [97]) using non-differentially expressed genes (base mean > 10 and P_{adj} > 0.05 obtained by likelihood ratio test in DESeq2) across all samples and time points as negative controls. Since the optimal parameters for the batch effect removal algorithm are not defined a priori, we employed a strategy of incremental increase of k values ($k=1,2,\ldots,n$), until we observed disruption of the PCA clustering of original data from the first batch of sequencing. To perform differential expression analysis, the obtained matrix of batch effect coefficients was further supplied to the design formula of the DESeq2 object, which was subsequently run using original count data. To plot 'batch-free' PCA plots, we used batch-corrected counts retrieved from the RUV package.

The list of 1-to-1 orthologues between the four fungal species was obtained from the CGD. For interspecies gene expression comparisons, the raw read counts for each fungal species were normalized by gene length and library size. GO term enrichment analysis was performed using clusterProfiler v.3.14.3 (ref. [98]). GO enrichment plots were produced with the dotplot function using showCategory set to 10 (for human data) and 8 (for fungal data) for better plot readability (the full list of GO enrichments is available in Supplementary File 9). Adjustment of P values was done using the Benjamini–Hochberg procedure. GO information for fungal species was obtained from the CGD, while for human data we used the Bioconductor package Genome wide annotation for Human (org.Hs.eg.db) v.3.10.0 in R[99].

We assessed the patterns of host–pathogen gene coexpression across the infections using the weighted correlation network analysis approach implemented in WGCNA v.1.69 (ref. [100]). For each infection, we combined fungal and corresponding human data at all available time points of infection, excluding

the data for the *C. albicans ece1*Δ/Δ mutant. As recommended by the package developers, we selected genes that had 10 or more counts in more than 90% of samples for downstream analysis. As expression levels, we used variance-stabilized read count data obtained using the vst function of DESeq2. Before the actual network construction, we first selected the β power values using the pickSoftThreshold function implying an unsigned network. The minimum β values reaching 80% of scale-free network topology, namely 12, 20, 7 and 22 for infections with *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*, respectively, were used for downstream analysis. After network construction, we inferred modules (that is, highly interconnected clusters of genes) in the WGCNA networks using 1-topology overlap matrix values at the 0.25 hclust tree cut-off and identified eigengenes (that is, the first principal component of each module). For each identified module, we performed GO term enrichment analysis of fungal and host genes using clusterProfiler (Supplementary File 10), selecting the top 3 enrichments with the lowest P_{adj} values. Then, the fungal gene content of each module of a given fungal species was compared against those of all modules of the other three species taking into account 1-to-1 orthology information (Extended Data Fig. 3). This analysis was done for modules that contained at least one fungal gene. Similarity between fungal gene contents of two given modules was defined as the intersection of the fungal gene lists of these modules divided by the union of these gene lists.

All custom calculations and visualizations were performed in R v.3.6.1 using various packages (all packages and their versions are available at our GitHub page https://github.com/Gabaldonlab/Host-pathogen_interactions).

Statistics and reproducibility. Experiments were performed in biological triplicates ($n=3$) with 3 different donors (neutrophil cytokine release) or 3 independent experiments. The growth curve experiments (Fig. 1b) were performed five times to ensure reproducibility. Only the mitochondrial aspect ratio was calculated based on 1 biological replicate (Fig. 4d) but multiple mitochondria were measured for each condition ($n>80$). All microscopy findings were reliably reproduced. Data were analysed using Prism 8 (GraphPad Software). Values are presented as the mean ± s.d. All the ratio data were log-transformed as indicated before statistical analysis in Prism and compared to 0 (uninfected/non-transfected/non-treated control) using a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (Figs. 1b and 6b,d) or Dunnett's multiple comparisons test (Figs. 4e–g, 5c and 6a and Extended Data Fig. 5c,d), Kruskall–Wallis test with two-sided Dunn's multiple comparison test (Fig. 4d), two-tailed one-sample *t*-test (Fig. 5a) or two-way ANOVA and Sidak's multiple comparisons test (Fig. 5b,d). Statistical significance is indicated in the figures as $^{*}P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$ and $^{****}P\leq0.0001$. The exact *P* values are provided in the Source data.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available within the paper and its Supplementary Information. All relevant data, including further image and processed data are available by request from the corresponding authors, with the restriction of data that would compromise the confidentiality of blood donors. Raw sequencing data have been deposited in the Sequence Read Archive under accession nos. SRR10279972–SRR10280067. Mapped data from the four *Candida* species can be mined and browsed at Candidamine (http://candidamine.org/candidamine/begin.do); the gene read counts from all samples can be found in our GitHub page https://github.com/Gabaldonlab/Host-pathogen_interactions along with the data analysis scripts for results reproducibility. Publicly available datasets/databases used in the study can be accessed at: Ensembl (https://www.ensembl.org/index.html); RefSeq (https://www.ncbi.nlm.nih.gov/refseq/); CGOB (http://cgob.ucd.ie/); NCBI FTP site (https://www.ncbi.nlm.nih.gov/home/download/); CGD (http://www.candidagenome.org/); and Genome wide annotation for Human (https://bioconductor.org/packages/release/data/annotation/html/org.Hs.eg.db.html). Source data are provided with this paper.

Code availability

All transcriptome data analysis results, including figures, extended data and supplementary materials are fully reproducible using the scripts provided at our GitHub page https://github.com/Gabaldonlab/Host-pathogen_interactions.

Received: 30 March 2020; Accepted: 1 February 2021;
Published online: 22 March 2021

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Acknowledgements

M.P., H.H., E.I., J.O.P., T.G., G.B. and B.H. received funding from the European Union Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant no. 642095 (OPATHY). B.H. also received support from the German Research Foundation within the Collaborative Research Centre/Transregio 124 FungiNet (project C1). M.S.G. was supported by the German Research Foundation Emmy Noether Programme (project no. 434385622/GR 5617/1-1). We acknowledge the support of the Spanish Ministry of Science, Innovation and Universities (grant no. PGC2018-099921-B-I00) to the European Molecular Biology Laboratory partnership, the Centro de Excelencia Severo Ochoa and the CERCA Programme/Generalitat de Catalunya.

We thank C. Kämnitz from the Electron Microscopy Center in Jena for the sample preparation for TEM. The schematic models in Figs. 4–6 were created with images adapted from Servier Medical Art (Servier).

Author contributions

M.P. performed all the laboratory experiments (except for TEM), analysed the data, wrote the manuscript and prepared the figures. H.H. performed all the bioinformatics analyses, wrote the manuscript and prepared the figures. E.I. and J.O.P. performed the infection experiments for RNA-seq and edited the manuscript. S.S.L. performed the growth curve and flow cytometry experiments and helped with the mtDNA depletion set-up, including the data analysis. T. Kalkreuter performed additional RT–qPCR experiments. S. Müller and T. Kamradt contributed to the additional mitochondrial phenotypic assays and data interpretation. E.S. and B.Q. performed the TEM experiments, analysed the data and edited the manuscript. M.S.G., S. Mogavero, S.B. and G.B. designed the experiments and edited the manuscript. B.H. and T.G. conceived and designed the study and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41564-021-00875-2.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41564-021-00875-2.

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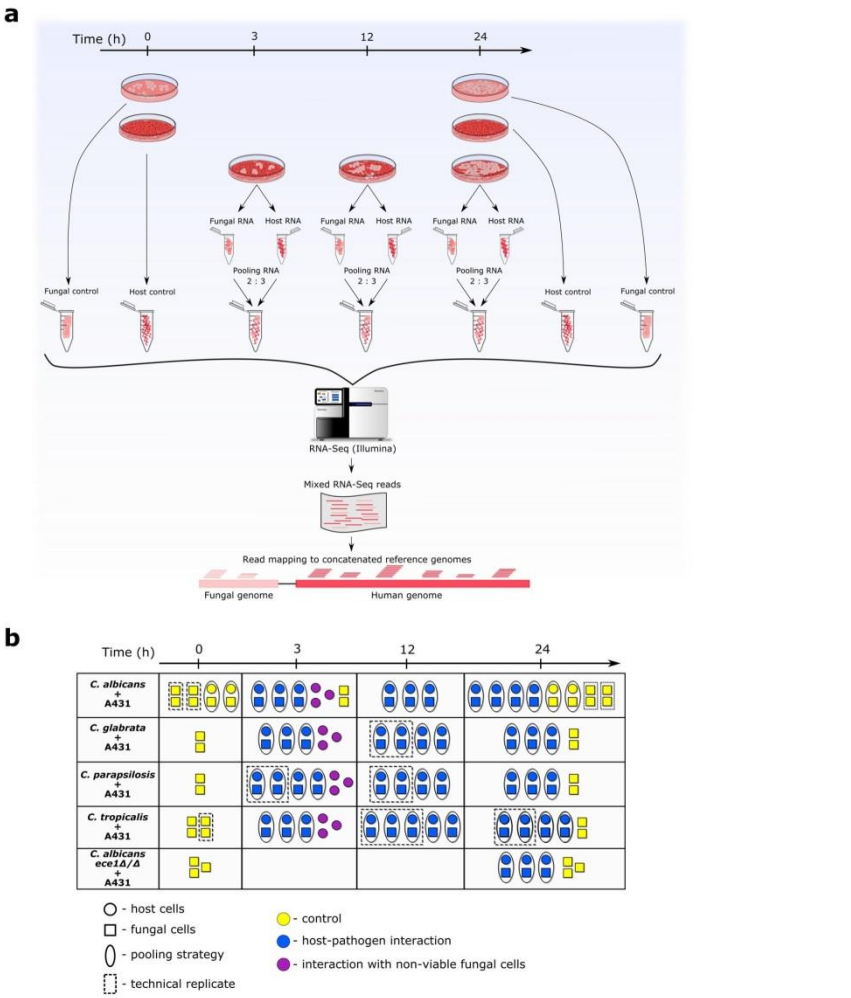
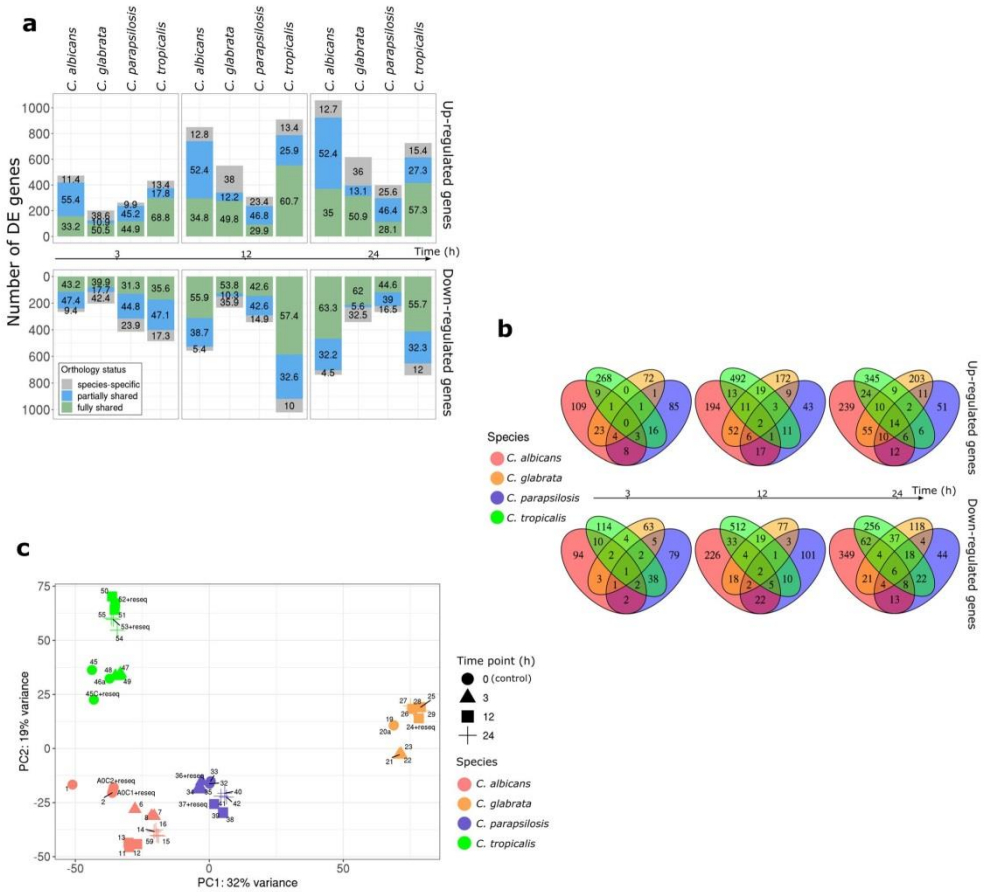
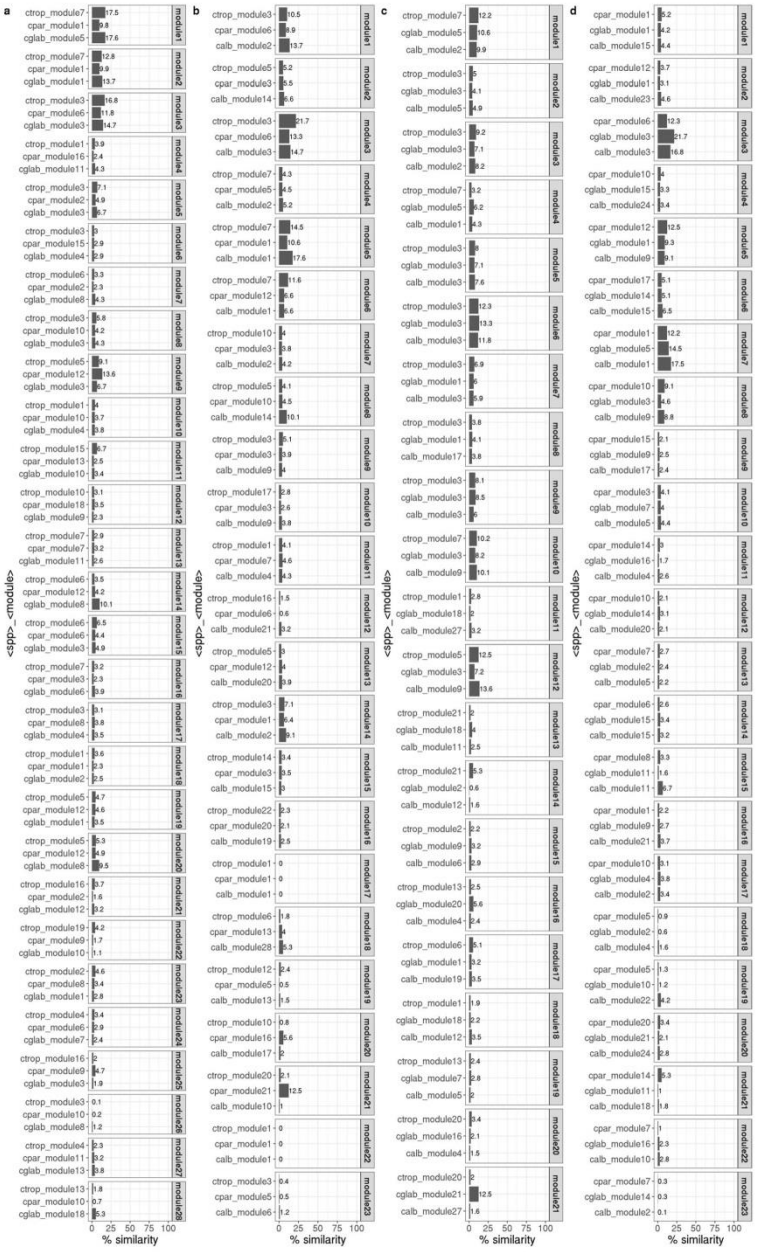
Peer review information *Nature Microbiology* thanks Elaine Bignell, Robert Watson and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.



Extended Data Fig. 1 | Overall experimental design of the current study. a, Schematic representation of the experimental design. Each *Candida* species was co-cultivated with host cells. Controls included samples at 0 and 24 h for both host and yeasts alone. At the indicated time points of infection, fungal and host RNAs were independently extracted and subsequently combined (pooled) at a 2:3 fungus-to-host ratio into one sample for library preparation and sequencing. Sequencing data were mapped to a concatenated host and fungal reference genome. **b**, Schematic representation of the entire study including all samples. Each symbol corresponds to a sequenced sample (or technical replicates of the same sample). Host samples are depicted with circles; *Candida* samples are depicted with squares; the strategy for combining (pooling) human and fungal RNAs in the same sequencing library is shown with ovals surrounding the corresponding samples; technical replicates (that is the same sequencing library sequenced several times) are surrounded with dashed rectangles. Control samples are depicted in yellow; interacting host and fungal samples are depicted in blue; host samples interacting with non-viable fungal cells are depicted in purple. Each row indicates the samples for each human-yeast interaction experiment.

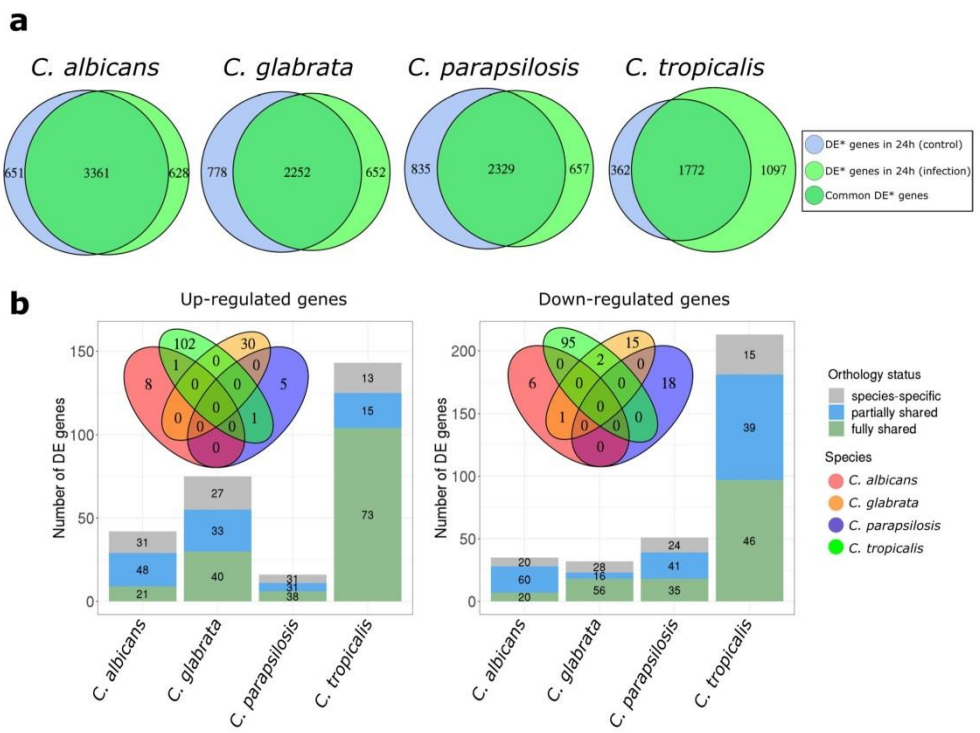


Extended Data Fig. 2 | Distinct patterns of transcriptome profiles of the four Candida species upon interaction with human epithelial cells. a, Distribution of fully shared, partially shared and species-specific differentially expressed (DE) genes across the course of infections. Numbers on bar plots indicate the percentage (%). **b**, Venn diagrams of DE genes (only 1-to-1 orthologs) in four *Candida* species at each time point. **c**, PCA biplot based on expression levels of orthologous genes across *Candida* species, demonstrating a species-specific stratification of transcriptomic profiles of the four fungal pathogens; Labels of the data points correspond to sample identifiers, where 'reseq' indicates that the sample was sequenced more than once (see Supplementary Table 1 for details).

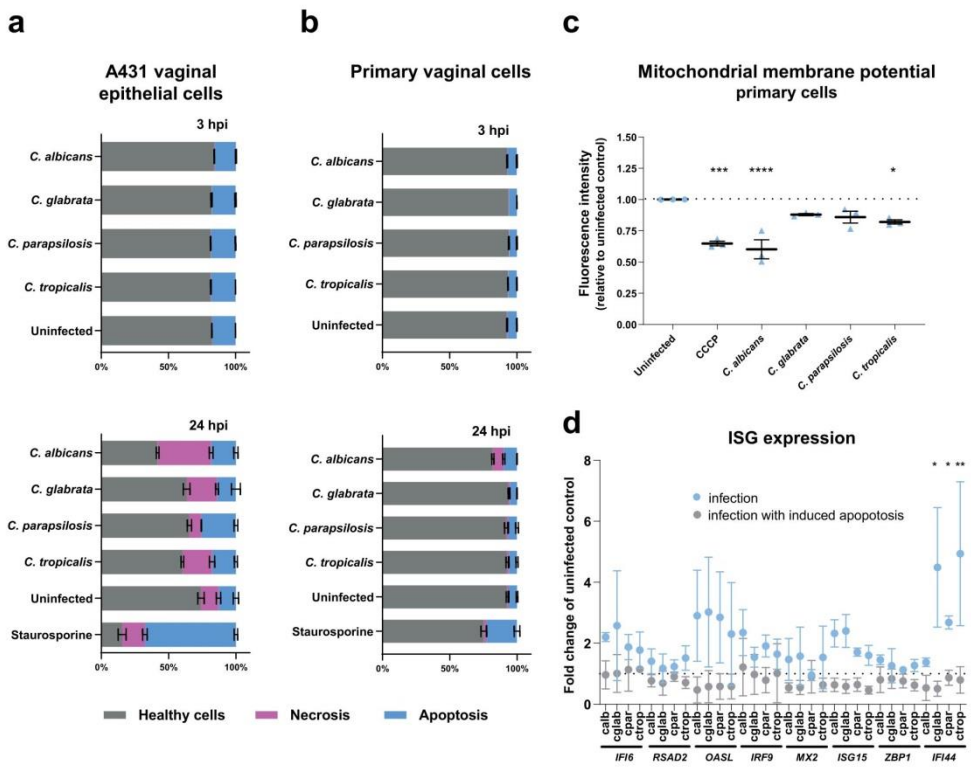


Extended Data Fig. 3 | See next page for caption.

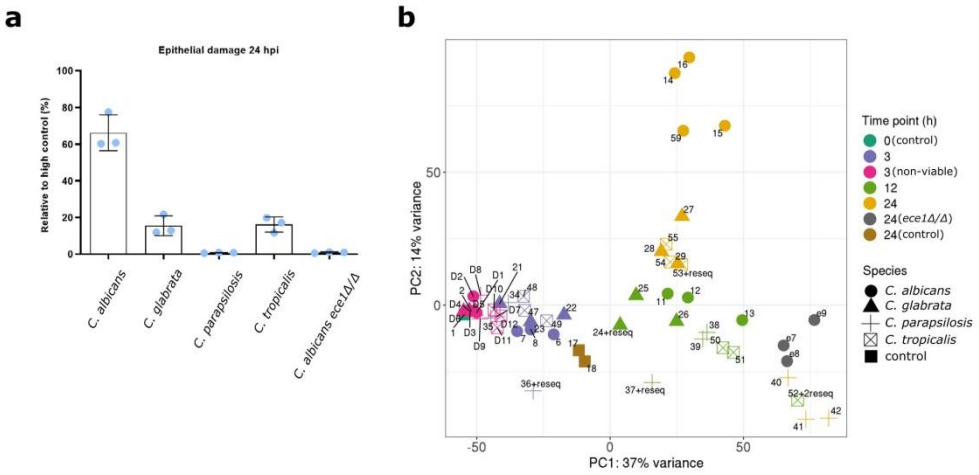
Extended Data Fig. 3 | Comparison of orthologous gene content similarities between co-expressed gene modules in different yeast species. a, Comparison of *C. albicans* modules against modules of other species. **b**, Comparison of *C. glabrata* modules against modules of other species. **c** Comparison of *C. parapsilosis* modules against modules of other species. **d**, Comparison of *C. tropicalis* modules against modules of other species. Each box represents a module of a given species (reference module); the title of a box represents the reference module name. Each reference module is compared with all modules of other three species, and the modules of other species with the highest similarity to the reference module are plotted with horizontal bars, representing level of similarity (in %). Labels of the horizontal bars indicate <species name>_<module name>. 'calb' denotes *C. albicans*, 'cglab' - *C. glabrata*, 'cpar' - *C. parapsilosis*, 'ctrop' - *C. tropicalis*. The level of similarity refers to the fraction (in %) of shared one-to-one orthologous genes between two given modules, defined as the intersection of gene lists of orthologs of two modules divided by the union of these gene lists.



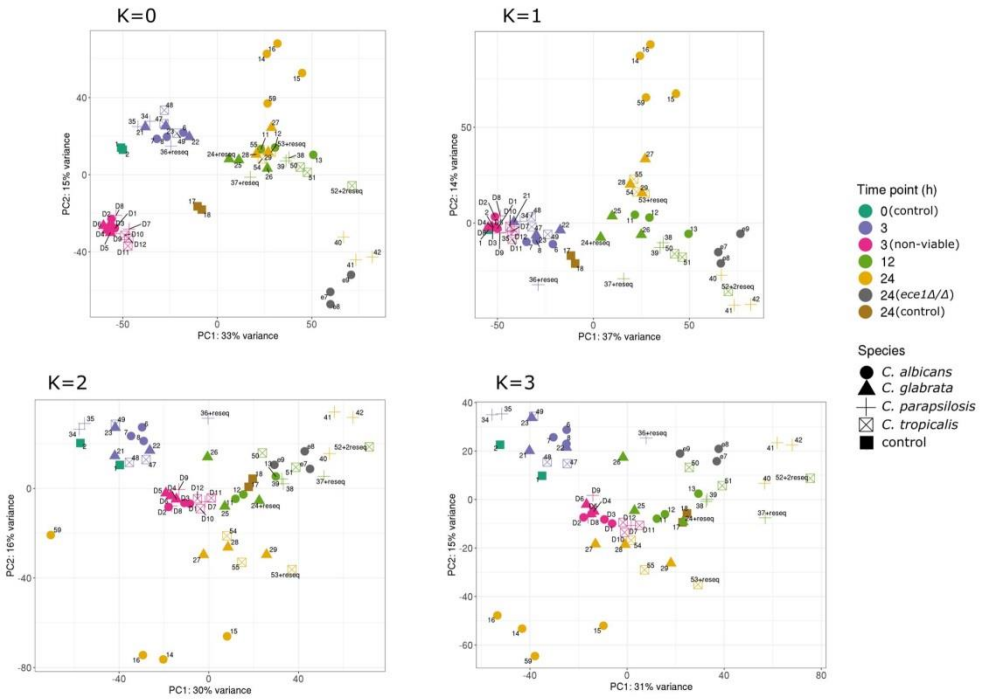
Extended Data Fig. 4 | Infection-specific differentially expressed (DE) genes of Candida species. a, Venn diagrams indicating similarities and differences of fungal DE* genes in culture medium only (control) and in response to epithelial cells (infection). *To identify infection-specific genes with a higher stringency, we applied filters of $|\log_2$ fold change$| > 0$ and $p_{adj} < 0.01$. For the downstream analysis of identified genes, we used a filtering of $|\log_2$ fold change$| > 1.5$ and $p_{adj} < 0.01$ for consistency with other results. Differential expression analysis was done using DESeq2 v. 1.26.0 and comparisons against time point 0 were done using the two-sided Wald test. **b**, Distribution of infection-specific fungal genes across the studied *Candida* pathogens. Bar plots demonstrate the distribution of partially shared, fully shared, and species-specific genes. Numbers on bar plots indicate the percentage (%). Venn diagrams depict numbers of fully shared genes (1-to-1 orthologs) across species.



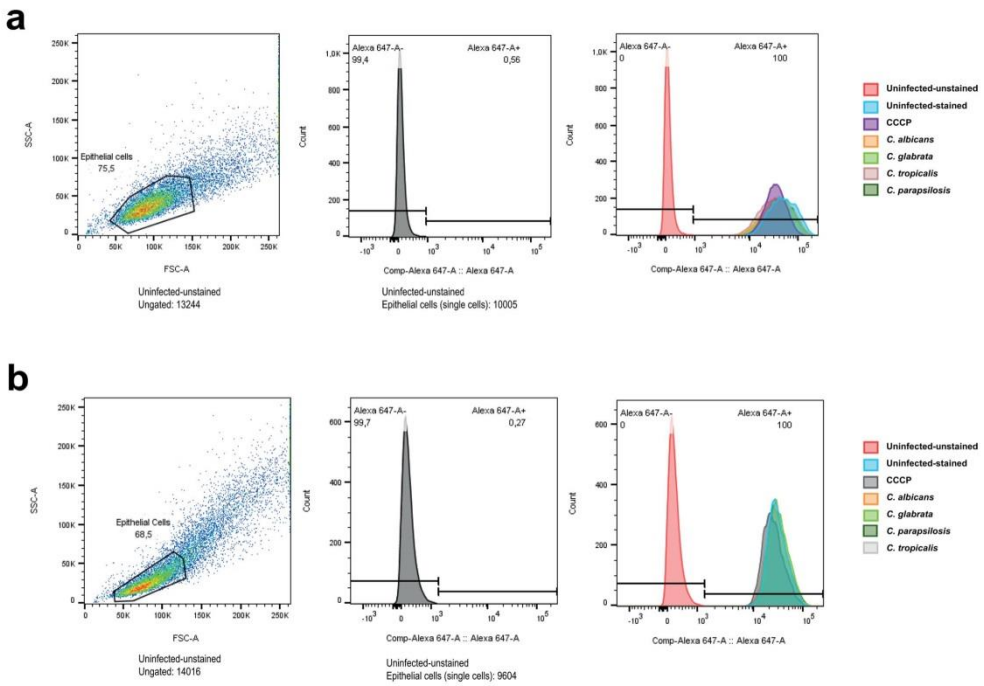
Extended Data Fig. 5 | Candida species induce type I interferon signalling independently of apoptosis. The proportion of healthy, necrotic, and apoptotic vaginal epithelial cells (ECs) 3 and 24 hours post-infection (hpi) with *Candida* in (**a**) A431 vaginal ECs used throughout this study and (**b**) primary vaginal ECs. Treatment with 1.2 μM staurosporine was used as a positive control. **c**, Mitochondrial membrane potential change of primary vaginal ECs at 1 hpi, positive control CCCP 100 μM. **d**, Relative expression (RT-qPCR) of selected Interferon-Stimulated Genes (ISGs) in *C. albicans*-infected ECs where apoptosis was induced with 1.2 μM staurosporine at 3 hpi. All values are presented as mean ± SD of n = 3 independent experiments. Statistical significance is indicated as: *, $p \leq 0.05$; ***, $p \leq 0.001$; ***, $p \leq 0.0001$ (one-way ANOVA with Dunnett's multiple comparisons test (**c-d**).; 'calb' denotes *C. albicans*, 'cglab' - *C. glabrata*, 'cpar' - *C. parapsilosis*, 'ctrop' - *C. tropicalis*.



Extended Data Fig. 6 | Human transcriptome profiles response to fungal damage. a, Levels of LDH release by epithelial cells upon the damage by four fungal pathogens 24 hpi. All values are presented as mean ± SD of n = 3 independent experiments. **b**, PCA plot of human samples interacting with non-viable and viable fungal species, including *C. albicans ece1*Δ/Δ. The plot is obtained using the RUVg function of RUVseq with k = 1 (see Extended Data Fig. 7 for plots with alternative k values). Labels of the data points correspond to sample identifiers, where 'reseq' indicates that the sample was sequenced more than once (see Supplementary Table 1 for details). 'non-viable' indicates host samples interacting with non-viable fungal cells; '*ece1*Δ/Δ' indicates host samples interacting with *C. albicans ece1*Δ/Δ.



Extended Data Fig. 7 | Human transcriptome response assessed with different parameters of batch effect correction. PCA plots of human samples interacting with fungal cells obtained using k = 0, 1, 2, 3 values of RUVseq package for batch effect correction. Labels of the data points correspond to sample identifiers, where 'reseq' indicates that the sample was sequenced more than once (see Supplementary Table 1 for details). 'non-viable' indicates host samples interacting with non-viable fungal cells; '*ece1*Δ/Δ' indicates host samples interacting with *C. albicans ece1*Δ/Δ.



Extended Data Fig. 8 | Applied gating strategies across flow cytometry experiments for epithelial cells. a, A431 cells (linked to Fig. 4e) and (**b**) primary vaginal cells (linked to Extended Data Fig. 6c). First, 10^4 events were analyzed based on their side scatter area (SSC-A) vs. forward scatter area (FSC-A). For further analysis, single cells were selected based on forward scatter height (FSC-H) vs. forward scatter area (FSC-A). MitoTracker® Deep Red FM signal was measured using detection channel Alexa 647-A. The unstained population was taken as a reference to determine the median fluoresce intensity of all samples (depicted as histogram Alexa 647-A- and Alexa 647-A+). The ratio from the median intensity of the stained/uninfected cells and unstained/uninfected cells was used as a reference to obtain the results of the infected samples shown in the manuscript figures.

6.3 Manuscript III

Transient mitochondria dysfunction confers fungal cross-resistance between macrophages and fluconazole

Sofía Siscar-Lewin, Toni Gabaldón, Alexander M. Aldejohann, Oliver Kurzai, Bernhard Hube, Sascha Brunke

mBio. 2021. DOI: 10.1128/mBio.01128-21

Summary:

In microbes, the loss or inactivation of antivirulence genes is an evolutionary signature of their adaptation to a pathogenic lifestyle. *Candida glabrata* is an important opportunistic pathogen related to the baker's yeast, *Saccharomyces cerevisiae*. Unlike *C. albicans,* the most highly pathogenic *Candida* species, *C. glabrata* does not form hyphae and elicit epithelial damage. Its main pathogenic traits are its intrinsic high level of azole resistance and its ability to persist within phagocytes. During *C. glabrata*'s evolution as a pathogen, the mitochondrial DNA polymerase gene, *CgMIP1*, has been under positive selection. Here it is shown that *CgMIP1* deletion triggers the respiratory-deficient *petite* phenotype through the loss of mitochondrial DNA and, subsequently, loss of mitochondrial function. Despite its slow growth, yeasts with the *petite* phenotype show increased azole and ER stress resistance, and survive better after phagocytosis. The same phenotype is induced by fluconazole and by exposure to macrophages, conferring a cross-resistance between antifungals and immune cells. The macrophages-derived *petite* can revert back to wild type growth once outside the phagosome, which confers the yeast a better fitness in competitive conditions. The *petite* phenotype can also be found in clinical isolates. This work suggests that the *petite* phenotype can be advantageous to *C. glabrata* during infection, and potentially be a relevant cause for the development of azole resistance in the clinics. Mitochondrial function may thus be considered a potential antivirulence factor.

The candidate is:

☒ First author ☐ Second author ☐ Corresponding author ☐ Coauthor

Estimated authors' contributions:

Author	Conception	Data analysis	Experimental	Writing	Provision of the material
SSL	70%	90%	90%	68%	84%
TG				1%	5%
AMA				1%	5%
OK					1%
BH	10%			10%	
SB	20%	10%	10%	20%	5%

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		Editor:	Section: **Research Article**	Designation:	

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RESEARCH ARTICLE

AQ: A

Transient Mitochondria Dysfunction Confers Fungal Cross-Resistance against Phagocytic Killing and Fluconazole

AQ: au Sofía Siscar-Lewin,[a] Toni Gabaldón,[b,c,d] Alexander M. Aldejohann,[e] Oliver Kurzai,[e] Bernhard Hube,[a,f] Sascha Brunke[a]

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ABSTRACT Loss or inactivation of antivirulence genes is an adaptive strategy in pathogen evolution. *Candida glabrata* is an important opportunistic pathogen related to baker's yeast, with the ability to both quickly increase its intrinsic high level of azole resistance and persist within phagocytes. During *C. glabrata*'s evolution as a pathogen, the mitochondrial DNA polymerase CgMip1 has been under positive selection. We show that *CgMIP1* deletion not only triggers loss of mitochondrial function and a *petite* phenotype, but increases *C. glabrata*'s azole and endoplasmic reticulum (ER) stress resistance and, importantly, its survival in phagocytes. The same phenotype is induced by fluconazole and by exposure to macrophages, conferring a cross-resistance between antifungals and immune cells, and can be found in clinical isolates despite a slow growth of *petite* strains. This suggests that *petite* constitutes a bet-hedging strategy of *C. glabrata* and, potentially, a relevant cause of azole resistance. Mitochondrial function may therefore be considered a potential antivirulence factor.

IMPORTANCE *Candida glabrata* is an opportunistic pathogen whose incidence has been increasing in the last 40 years. It has risen to become the most prominent non-*Candida albicans Candida* (NCAC) species to cause candidemia, constituting about one-third of isolates in the United States, and steadily increasing in European countries and in Australia. Despite its clinical importance, *C. glabrata*'s pathogenicity strategies remain poorly understood. Our research shows that loss of mitochondrial function and the resulting *petite* phenotype is advantageous for *C. glabrata* to cope with infection-related stressors, such as antifungals and host immune defenses. The (cross-)resistance against both these factors may have major implications in the clinical outcome of infections with this major fungal pathogen.

KEYWORDS fungal infection, *petite*, cross-resistance, antivirulence

Human-pathogenic fungi remain an underestimated threat in global health, and the mortality rates of fungal infections worldwide are higher or similar to deaths due to malaria or tuberculosis (1, 2). *Candida* species are among the most important human fungal pathogens and cause millions of mucosal and life-threatening systemic infections each year (1). *Candida glabrata* has become the second most common *Candida* species for immunocompromised patients, surpassed only by *C. albicans* as the primary cause of candidiasis (3). However, most of the well-characterized pathogenicity mechanisms of *C. albicans* are not shared by *C. glabrata*, and unlike the former, *C. glabrata* does not cause significant host cell damage or elicit strong host immune responses (4). Among the main clinically relevant attributes and pathogenic traits of *C. glabrata* are rather a high intrinsic resistance to azole antifungals and an ability to survive for a long

Citation Siscar-Lewin S, Gabaldón T, Aldejohann AM, Kurzai O, Hube B, Brunke S. 2021. Transient mitochondria dysfunction confers fungal cross-resistance against phagocytic killing and fluconazole. mBio 12: e01128-21. https://doi.org/10.1128/mBio.01128-21.

Editor Deborah A. Hogan, Geisel School of Medicine at Dartmouth

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This article is a direct contribution from Bernhard Hube, a Fellow of the American Academy of Microbiology, who arranged for and secured reviews by James Kronstad, University of British Columbia, and Hubertus Haas, Medical University of Innsbruck.

Received 20 April 2021
Accepted 26 April 2021
Published

ID: Amit.Kumar1Time: 15:31I Path: //mumnasprod/home$/joshis$/SM-MBIO210278

11/5/21 | 15:31 | 4/Color Fig: 1,2,3,4,6,7 | ArtID: | DOI:10.1128/mBio.01128-21 | CE: KGLL-rms

time and replicate within mononuclear phagocytes (4–6). Its redundant antioxidative stress mechanisms, combined with its ability to modify the phagosomal pH, may partially account for the remarkable ability to survive phagocytosis by macrophages (7–9). These facts have led to the speculation that *C. glabrata* may take advantage of these immune cells to succeed as a pathogen and disseminate within the host (6).

Among the strategies that confer pathogenicity, the loss or inactivation of certain genes, termed antivirulence genes, is common in pathogenic microorganisms (10); cellular pathways and functions that are normally advantageous for the microbe can become superfluous or even disadvantageous under infection conditions, and the loss or inactivation of their encoding genes becomes adaptive during infection. Several examples of such antivirulence factors are known in human-pathogenic fungi, and many more are likely to exist (11).

C. glabrata is more closely related to the brewer's yeast *Saccharomyces cerevisiae* than to *C. albicans* (12) and clusters with members of the *Nakaseomyces* group, a genus that includes other environmental and human-associated species (5). In a systematic genomic comparison within this group, four genes showed hallmarks of positive selection in *C. glabrata* (5). These genes exhibit a relatively high ratio of nonsynonymous to synonymous mutations (d_N/d_S), indicating positive selection during the diversification of *C. glabrata* as a species. Therefore, they might be involved in *C. glabrata*'s specific adaptation to the human host. The gene with the highest d_N/d_S ratio (3.40) among them is *CgMIP1*, an orthologue of a mitochondrial DNA (mtDNA) polymerase in *S. cerevisiae* (5).

mtDNA encodes subunits of the respiratory complexes, which are involved in the production of ATP during oxidative phosphorylation. The consequences of mtDNA loss have been well described in *S. cerevisiae* and *C. glabrata*, which, unlike other pathogenic yeasts such as *C. albicans* or *Cryptococcus neoformans* (13, 14), are known as *petite*-positive yeasts, since they are able to grow without mtDNA. The *petite* phenotype is characterized by loss of mitochondrial function due to complete (rho^0) or partial loss (or presence of deleterious mutations) of mtDNA (rho^-) (15). This phenotype is characterized by the namesake small colonies, slow growth, inability to use nonfermentable carbon sources, activation of the transcription factor *PDR1*, and upregulation of its targets *CDR1* and *CDR2*, which code for ABC efflux pump transporters (13). This upregulation confers high resistance to azole antifungals (16–18). Indeed, the *petite* phenotype can be obtained by incubation with high concentrations of azole or ethidium bromide (17, 19, 20). Ethidium bromide is known to inhibit mtDNA synthesis and degrade the existing mtDNA (20), but how azoles trigger mitochondrial dysfunction is not entirely clear. Azole treatment is known to trigger a temporary loss of mitochondrial function (21), and the few clinical *petite* strains of *C. glabrata* described so far have been mainly isolated from azole-treated patients (19, 22). One of these isolates has been further characterized (23). Surprisingly, these slow-growing isolates showed increased virulence in an animal infection model (23). However, when its parental strain was made *petite* by ethidium bromide treatment, its virulence was instead reduced. The same was found in another study using an ethidium bromide-induced *petite* (24). Thus, the clinical relevance of the *petite* form is still unclear, and its identification from patient samples may even be hindered by its long generation time.

This study investigates the relevance of the presence and absence of mitochondrial function for *C. glabrata*'s adaptation to the host and its pathogenic potential, as well as the potential role of *CgMIP1* for switching between *petite* and non-*petite* phenotypes. Deletion of *MIP1* results in *petite* forms, but in contrast to the *S. cerevisiae Scmip1*Δ mutant, *C. glabrata Cgmip1*Δ survives phagocytosis by macrophages significantly better than wild-type cells. Importantly, the *C. glabrata petite* phenotype is directly induced in wild-type strains by phagocytosis and leads to increased azole resistance, but also vice versa, with azole-induced *petites* resisting phagocytosis better. This indicates a clinically important positive feedback between two relevant phenotypes, resistance to macrophages and azoles. The clinical relevance of this phenomenon was further corroborated by the detection of a number of *petite* strains in clinical samples.

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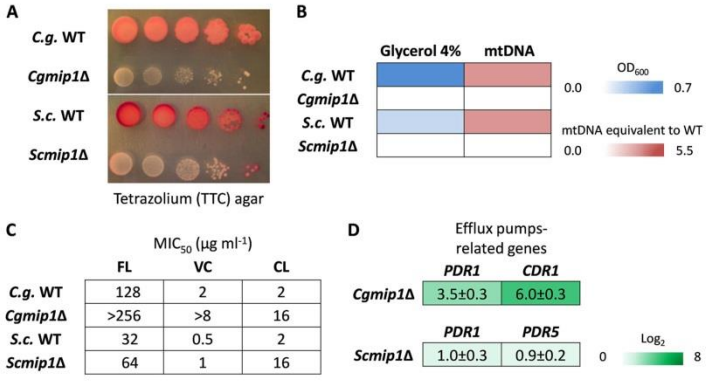

COLOR

FIG 1 Both *Cgmip1*Δ and *Scmip1*Δ show typical *petite* phenotypes. (A) Small colonies and loss of mitochondrial reductive power, as indicated by lack of tetrazolium dye reduction; (B) lack of growth in alternative carbon sources such as glycerol and absence of mitochondrial (mt) DNA as determined by optical density and qPCR ($n = 3$ for each type of experiment, color by mean); (C) high resistance to azoles, including fluconazole (FL), voriconazole (VC), and clotrimazole (CL), and (D) overexpression of efflux pump-related genes (mean ± SD, $n = 3$ independent experiments with 3 technical replicates each). *C.g.*, *C. glabrata*; *S.c.*, ●●●.

AQ: 1

RESULTS

***MIP1* knockout mutants of *C. glabrata* and *S. cerevisiae* both show *petite* phenotypes but differ in their survival after phagocytosis.** Since the *MIP1* gene of *C. glabrata* seems to have been under selective pressure during the pathogen's evolution, we investigated its functions in virulence-related scenarios. First, we created a deletion mutant of *CgMIP1* (*Cgmip1*Δ) and compared it to a similar deletion of the orthologous gene in *S. cerevisiae* (*Scmip1*Δ). *ScMIP1* is known to encode a mitochondrial polymerase (*Saccharomyces* Genome Database [SGD], www.yeastgenome.org), and therefore, we first checked whether the mutants show the *petite* phenotype. As expected, both *Cgmip1*Δ and *Scmip1*Δ showed a phenotype typical for *petite* variants (16–18)—small colonies on agar plates and absence of reductive mitochondrial power (Fig. 1A), absence of mtDNA, and lack of growth in nonfermentable carbon sources (Fig. 1B). Moreover, they showed high resistance to azoles (Fig. 1C) and high expression of the efflux pump-related genes *PDR1* and *CDR1* (*PDR1* and *PDR5* in *S. cerevisiae*) (Fig. 1D), again typical features of *petite* variants. These results therefore show that, like its *S. cerevisiae* counterpart, *CgMIP1* likely encodes a mitochondrial DNA polymerase, and its deletion triggers loss of mtDNA, loss of mitochondrial function, and a *petite* phenotype in both species.

F1

To study a possible involvement of *MIP1* in processes relevant for virulence, we subjected *Cgmip1*Δ to phagocytosis by human monocyte-derived macrophages (hMDMs) and analyzed its survival after 3 and 6 h. At those time points, macrophages were lysed, and total CFU were counted after plating on yeast extract-peptone-dextrose (YPD) agar. A significantly higher survival rate of *Cgmip1*Δ was found at both times compared to both the wild-type control and to *Scmip1*Δ (Fig. 2A). In order to confirm that this increased number of surviving intracellular yeasts was indeed due to better survival and not due to differences in phagocytosis rate or internal replication, we measured both parameters. For phagocytosis rates, *Cgmip1*Δ and the wild type were incubated with hMDMs for 1 h, and CFU from supernatant and macrophage lysate were determined. *Cgmip1*Δ cells were taken up at a slightly higher rate compared to the wild type (Fig. 2B), which, however, alone cannot explain the stark increase in the number of intracellular *Cgmip1*Δ especially at 6 h; whereas the *petite* mutant is taken up 1.5

F2

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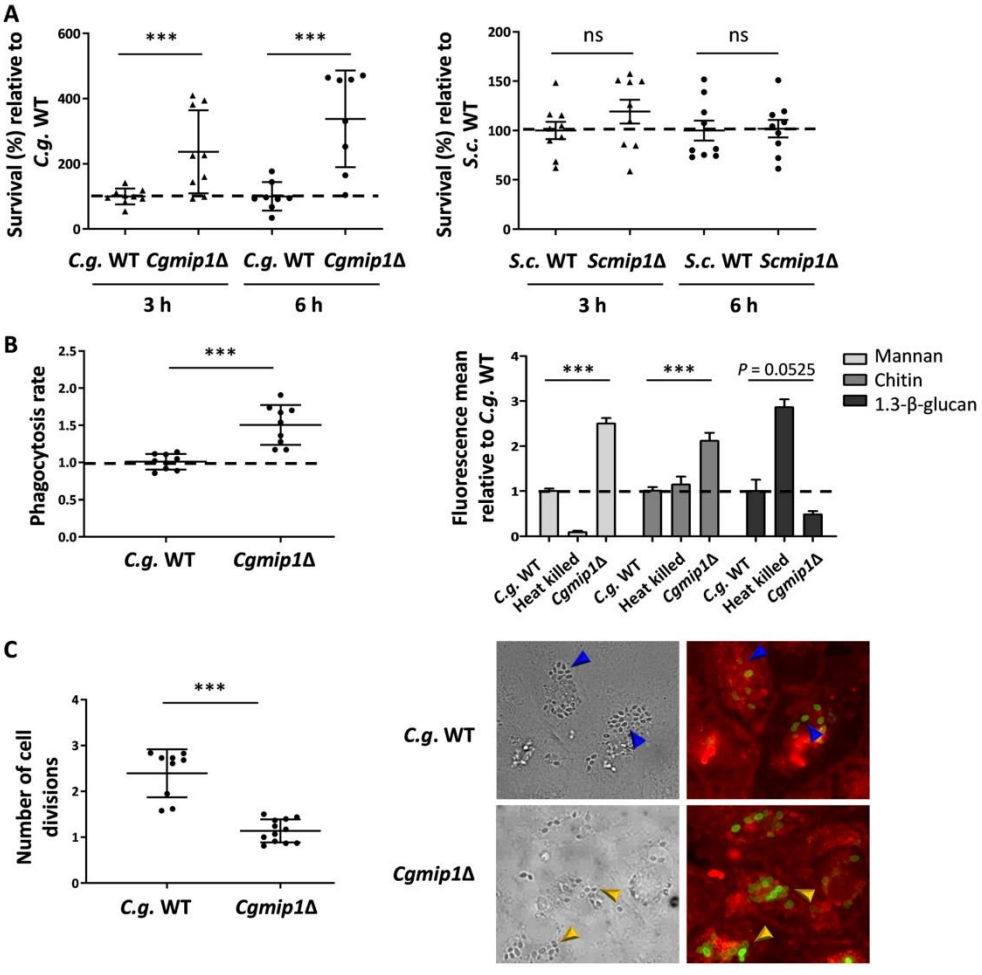

COLOR

FIG 2 *C. glabrata* and *S. cerevisiae petite* phenotypes differ in their survival after phagocytosis. (A) *Cgmip1*Δ survives phagocytosis by hMDMs much better than its parental wild type at early time points up to 6 h—in contrast to *Scmip1*Δ, which does not show any change in survival compared to its wild type (mean ± SD, $n = 9$ with 3 different donors in 3 independent experiments; each point represents the mean of 3 technical replicates). (B) *Cgmip1*Δ is taken up at a higher rate than the wild type by macrophages (mean ± SD, $n = 9$ with 3 different donors in 3 independent experiments; each point represents the mean of 3 technical replicates), and its accessible cell wall structures differ from the wild type (mean ± SD, $n = 3$ independent experiments with 3 technical replicates each). (C) In contrast to the wild type, *Cgmip1*Δ does not replicate within the phagosome as shown by FACS (left) and by the lack of FITC-unstained daughter cells (right). These are present in the wild type (blue arrows), in contrast to the mutant, which shows only mother cells (yellow arrows) (representative picture shown). Quantitative data are the mean ± SD; $n = 12$ with 3 different donors in 4 independent experiments; each point represents the mean of 3 technical replicates. Statistically significantly different values (unpaired, two-tailed Student's *t* test on log-transformed ratios) are indicated by asterisks as follows: ***, $P \leq 0.001$. *C.g.*, *C. glabrata*; *S.c.*, ●●●.

times more than the wild type, the survival of this mutant is up to four times more than that of the wild type after 3 h, and six times more at 6 h.

To gain more insight into the underlying reason for this slightly increased uptake of *Cgmip1*Δ, the exposure of cell wall components was measured by flow cytometry. Significantly higher exposure of mannan and chitin was observed (Fig. 2B), while

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exposure of $\beta(1\rightarrow3)$-glucan was slightly reduced. Higher surface mannan levels on yeast cells are known to increase the phagocytosis rate (25), and thus, our results show that mitochondrial dysfunction by deletion of *CgMIP1* affects cell wall composition in *C. glabrata*—in agreement with previous observations (24, 26)—and leads to changes in the phagocytosis rate. In addition, it has been reported that higher surface mannan seems to lead to a decreased killing of *C. glabrata* (27) in a yet unknown mechanism, from which *Cgmip1*Δ would benefit during phagocytosis. To also directly measure fungal replication within the macrophages, yeasts were fluorescein isothiocyanate (FITC)-stained and incubated with hMDMs for 6 h. This stain is not transferred to daughter cells, and we measured FITC-negative cells in the macrophage lysate by flow cytometry and also visualized them with fluorescence microscopy. According to our FACS data, *Cgmip1*Δ showed a much lower replication rate than its parental strain, and we did not observe any unstained daughter cells by microscopy (Fig. 2C). This is in accordance with the nutrient limitation in the phagosome, where only nonfermentable (and therefore *petite*-inaccessible) carbon sources (carboxylic acids, amino acids, peptides, *N*-acetylglucosamine, and fatty acids) are thought to be available (28–30). This also shows that the wild type is killed much more efficiently than the *petite* strains, resulting in a higher CFU count for the latter despite its lower replication rate within the phagosome.

These results indicate that, although *Cgmip1*Δ *petite* phenotype is engulfed faster and is largely unable to replicate inside the macrophage, it is killed significantly more slowly in the first hours of immune cell interaction, in clear contrast to nonpathogenic *S. cerevisiae*.

The *petite* phenotype emerges from the wild type after phagocytosis. Our data so far indicate a selective advantage of the *petite CgMIP1* deletion strain during initial interactions with macrophages, despite its inability to replicate within these cells. We therefore analyzed survival of *Cgmip1*Δ and the wild type during long-term residence within macrophages. For this experiment, yeasts were first incubated with macrophages for 3 h, the supernatant was removed, and the yeast-containing macrophages were then incubated for 7 days. Fungal survival was assessed by CFU counting from plated lysate at 3 h, 1 day, 4 days, and 7 days. *Cgmip1*Δ again showed higher survival up to 1 day of coincubation, in full support of our previous results (Fig. 3A). However, at later time points, *Cgmip1*Δ showed a significant decrease in survival. This may be explained by its inability to replicate within the phagosome, leading to a long-term disadvantage. Unexpectedly, we spotted many small colonies during incubation of the wild type. These colonies were more abundant across all observed time points compared to the inoculum, and it was especially true after 3 h and 1 day of incubation (the time points with the best survival of the *petite* strain), with an average frequency of 1.5×10^{-2} after 1 day (Fig. 3B). These colonies showed stable small colony formation and lack of growth in glycerol, typical features of the *petite* phenotype. Importantly, this frequency was higher than the spontaneous emergence of *petite* without macrophages at the same time points (Fig. 3B). In addition, some of the colonies which did not grow in glycerol gave rise to a respiratory-competent phenotype, i.e., returned to their original non-*petite* state, when they were plated again on complex medium, with an observed frequency of 5.6×10^{-2}.

We analyzed several of the stable wild-type-derived *petite* colonies and found—in the majority, but not all of them—a lack of detectable mtDNA. We selected three of these stable colonies from different experiments for further characterization of their *petite* phenotype (Fig. 3C). Besides lacking mtDNA and therefore functional mitochondria, these strains also showed high azole resistance with constitutive expression of efflux pump-related genes, although they were not exposed to azoles, similar to *Cgmip1*Δ (Fig. 3).

We hypothesized that phagosomal reactive oxygen species (ROS) production may have contributed to the loss of mitochondrial function (31, 32). We therefore incubated wild-type yeasts in RPMI medium with a sublethal concentration (10 mM) of H_2O_2 for

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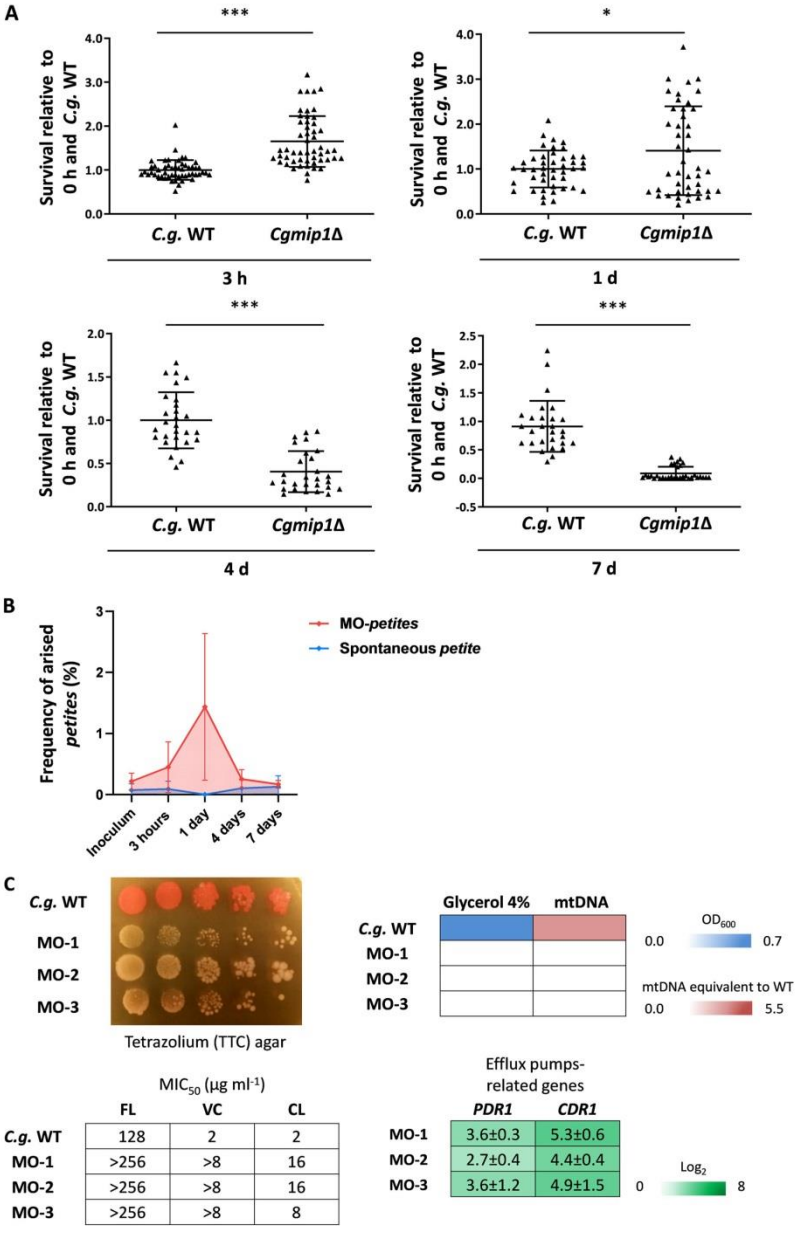

FIG 3 The *petite* phenotype emerges from the wild type after phagocytosis. (A) *Cgmip1*Δ shows a higher rate of survival up to 1 day of coincubation but loses this advantage over extended periods of intracellular existence ($n = 5$ with 1 different

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24 h and observed the emergence of small colonies at a low frequency, which did not grow in glycerol (data not shown).

These results indicate that the *petite* phenotype is adaptive within macrophages at early time points, but not at later times, probably due to the long period of starvation in the phagosome that prevents it from replicating. In agreement with this presumable advantage, the *petite* phenotypes arise from the respiratory-competent yeasts after 3 h to 1 day of phagocytosis, the same time period in which *Cgmip1*Δ shows a higher fitness. Importantly, these macrophage-induced *petites* can revert to a respiratory metabolism when grown again in the absence of stress.

The *petite* phenotype triggered by fluconazole increases survival of phagocytosis at early time points. It is known that azoles trigger (often temporary) mitochondrial dysfunction in *C. glabrata* (21, 33), which leads to fluconazole resistance through the upregulation of the efflux pump genes *CDR1* and *CDR2*, with the former being especially important in this resistance (16–18). In fact, *petite* mutants have very occasionally been isolated from patients undergoing fluconazole treatment (19, 22). Since our results showed an advantage of the genetically created *petite* strains after phagocytosis, we wondered whether fluconazole-induced *petites* share the same increased fitness. We therefore incubated wild-type yeasts for 8 h in RPMI medium with 8 μg/ml of fluconazole, half the concentration of the reported MIC_{50} for *C. glabrata* (34). Again, we observed the appearance of small colonies with a reversible and stable *petite* phenotype (Fig. 4A). When the stable strains were coincubated with macrophages for three and 6 h, all fluconazole-induced *petites* showed better survival in macrophages at both time points (Fig. 4B).

These results indicate a cross-resistance of the *petite* phenotype induced by and also protecting from both phagocytosis and fluconazole; exposure to fluconazole triggers a higher fitness of *C. glabrata* inside macrophages, and vice versa, fluconazole-resistant yeasts appear within macrophages.

***Cgmip1*Δ shows higher basal expression of stress response-related genes and grows better under ER stress.** To understand why switching to a *petite* phenotype increases intraphagosomal fitness, we measured macrophage ROS production and damage, as it is known that *C. glabrata* is able to inhibit or detoxify ROS within the phagosome and also to burst the immune cell (9, 27). However, we did not observe significant differences between the wild type and *petite* for these parameters (Fig. S1). Since a reduced stress resistance correlates with a reduced intracellular survival (27), we also measured the basal and induced expression of different stress-response genes and tested the resistance to infection-related stresses. *Cgmip1*Δ and the macrophage- and fluconazole-derived *petite* strains showed an increased basal expression of a range of environmental stress response genes (*HSP12*, *HSP42*, and *SGA1*) and cell wall stress-related genes encoding yapsins (*YPS1*, *YPS10*, and *YPS8*) (Fig. 5A). These genes have been shown to be highly upregulated in the *petite* isolate BPY41 and may be involved in its hypervirulence (23). The yapsin *YPS* gene family has been implicated in the survival inside macrophages (35), and we found that while the three *YPS* genes were upregulated in *C. glabrata*, in the *S. cerevisiae petite* mutant (which does not survive phagocytosis better than its wild type) the most similar yapsin genes (*YPS1* and *MKC7*) showed no increased basal transcript levels (Fig. S2A).

FIG 3 Legend (Continued)
donor in each of the 5 independent experiments; each point represents a single survival test). Statistically significantly different values (unpaired, two-sided Student's *t* test on log-transformed ratios) are indicated by asterisks as follows: *, $P \leq 0.05$; ***, $P \leq 0.001$. (B) Cells with the *petite* phenotypes arise from the wild type during coincubation with hMDMs (red) at a much higher rate than the spontaneous appearance of *petite* in RPMI (blue). The time points with the highest frequency of *petite* emergence correspond to the time point of increased fitness of *Cgmip1*Δ during phagocytosis (Red: $n = 5$ with 1 different donor in each of the 5 independent experiments and 4 technical replicates each. Blue: $n = 3$ in 3 different experiments with 6 technical replicates. Mean ± SD of the frequencies of *petite* emergence at each time point). (C) Phenotype characterization reveals the hMDMs-derived strains (MO-1 to -3) to be indeed *petite*. From the top left, small colony forming and lack of mitochondrial reductive power, lack of growth in alternative carbon sources, absence of mitochondrial (mt) DNA, high resistance to azoles (all $n = 3$ with mean values or representative picture shown) and overexpression of efflux pump-related genes (mean ± SD, $n = 3$ independent experiments with 3 technical replicates each). FL, fluconazole; VC, voriconazole; CL, clotrimazole; *S.c.*, ●●●.

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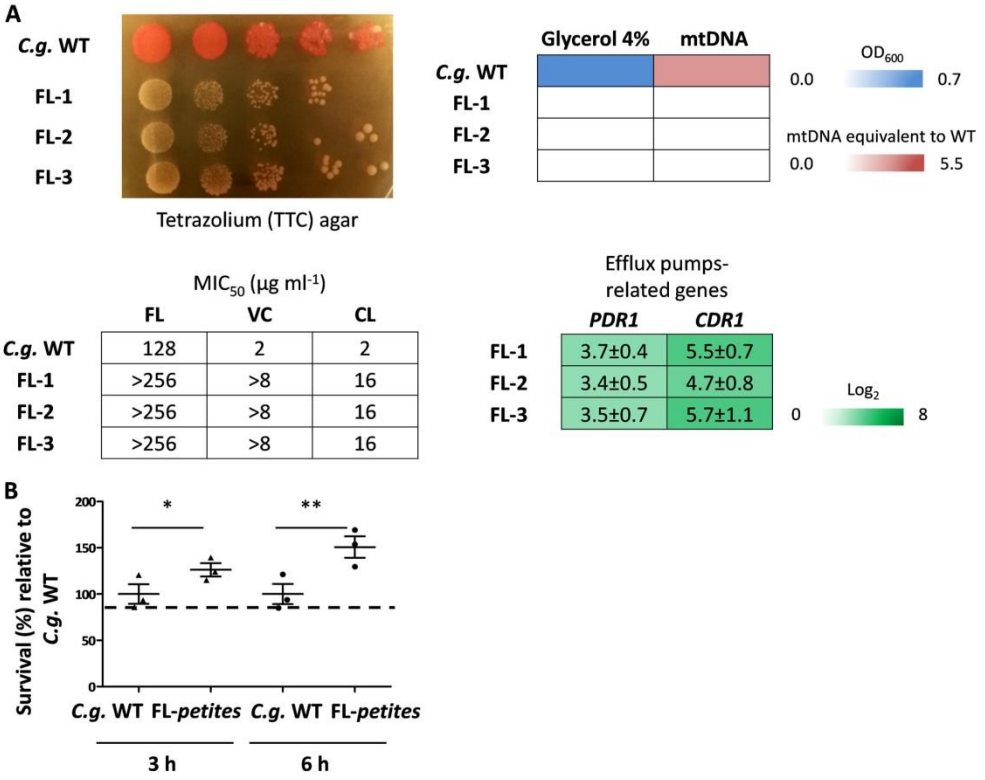

COLOR

FIG 4 The *petite* phenotype triggered by fluconazole increases survival of phagocytosis at early time points. (A) Fluconazole-induced *petites* show the *petite* phenotype similar to *Cgmip1*Δ—small colonies and lack of mitochondrial reductive power, lack of growth in alternative carbon sources, absence of mitochondrial (mt) DNA, high resistance to azoles (all $n = 3$ with mean values or representative picture shown), and overexpression of efflux pump-related genes (mean ± SD, $n = 3$ independent experiments with 3 technical replicates each). FL, fluconazole; VC, voriconazole; CL, clotrimazole. (B) Fluconazole-induced *petites* (FL-1 to FL-3) show better survival of phagocytosis at early time points (mean ± SD, $n = 3$ with 1 donor in 3 independent experiments; each point represents a mean of 3 different colonies per donor, and each colony has 3 technical replicates). Statistically significantly different values (unpaired, two-sided Student's *t* test on log-transformed ratios) are indicated by asterisks as follows: *, $P \leq 0.05$; **, $P \leq 0.01$. *C.g.*, *C. glabrata*.

We then tested *Cgmip1*Δ in different *in vitro* stress conditions, such as osmotic stress, metal stress, ER stress, oxidative stress, and cell wall stress, and found no relevant phenotype. Interestingly, *Cgmip1*Δ showed oxidative stress resistance comparable to the wild type (Fig. S2B), in contrast to mitochondrial mutants of *S. cerevisiae* that are known to be especially sensitive to H_2O_2 (36). Therefore, we discarded a decreased sensitivity to oxidative stress as a possible explanation for the higher survival of *Cgmip1*Δ within macrophages. However, *Cgmip1*Δ and the azole- and macrophage-induced *C. glabrata petites* showed better growth than their wild type under ER stress conditions (Fig. 5B), in contrast to the *petite* mutant of *S. cerevisiae* Sc*mip1*Δ (Fig. S2A). Since efflux pumps play a central role in azole resistance, we analyzed mutants lacking their main transcriptional activator Pdr1 (37, 38) in both the wild-type (*pdr1*Δ) and the *Cgmip1*Δ (*Cgmip1*Δ+*pdr1*Δ) background. As expected, both mutants grew poorly in increasing concentrations of fluconazole (Fig. S2C). However, under ER stress, the double mutant (lacking *CgMIP1* and *CgPDR1*) still exhibited significantly better growth than the single mutant and the wild type (Fig. S2C), showing that the *Cgmip1*Δ resistance to ER

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A

	Stress response-related genes			Cell wall integrity, adherence to mammalian cells, survival in macrophages and virulence		
	HSP12	*HSP42*	*SGA1*	*YSP1*	*YPS8*	*YPS10*
Cgmip1Δ	6.4±0.6	2.5±1.2	2.5±1.0	2.3±0.5	3.7±0.3	3.3±1
MO-1	3.9±0.9	3.7±0.2	2.8±0.5	2.0±0.3	1.4±0.1	2.7±0.1
MO-2	2.3±0.5	3.6±0.2	2.4±0.1	-0.3±0.1	-0.3±0.7	0.3±0.2
MO-3	1.0±0.6	2.5±1.2	2.5±0.7	2.4±0.3	1.7±0.0	2.9±0.1
FL-1	3.7±0.5	4.0±0.1	3.6±0.6	1.0±0.4	2.2±0.4	3.0±0.9
FL-2	3.7±0.6	3.4±1.7	3.2±1.3	-0.2±0.9	1.1±1.2	2.5±2.0
FL-3	4.2±0.9	4.1±0.2	3.5±0.7	1.4±0.7	2.0±0.6	3.1±1.0

Log$_2$ 0 — 8

B

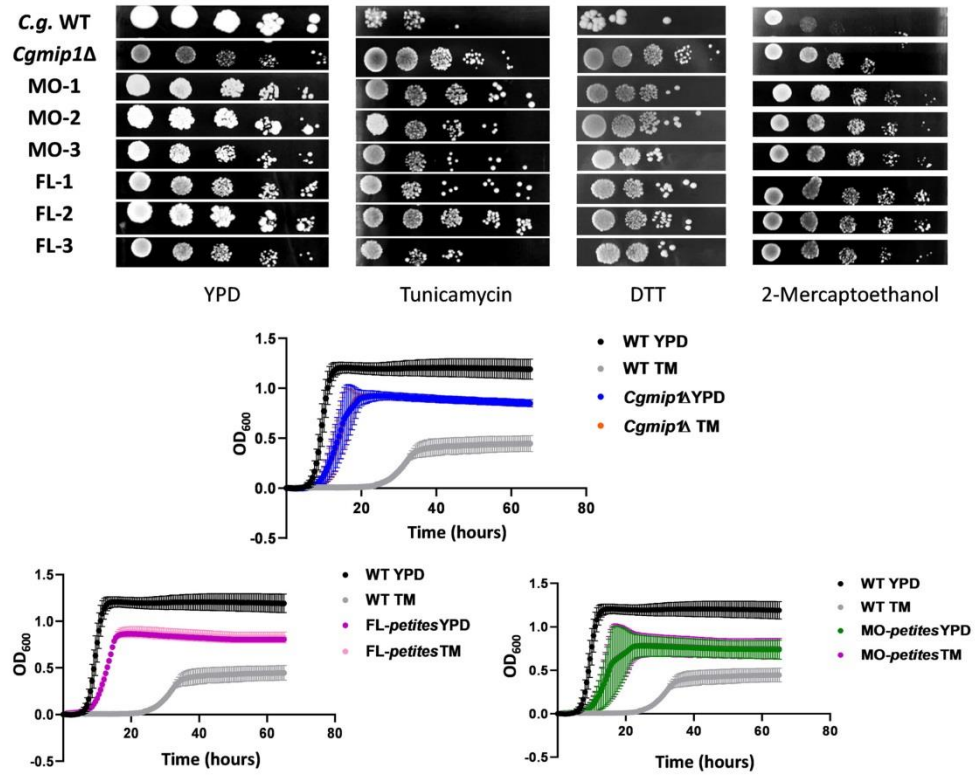

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FIG 5 *Cgmip1Δ* shows higher basal expression of stress response-related genes and grows better under ER stress. (A) *Petite* variants of *C. glabrata* show high constitutive expression of stress-response genes even under nonstressed conditions (YPD) (mean ± SD, $n = 3$ independent experiments with 3 technical replicates each), and (B) exhibit better growth than their wild type under different ER stresses on plates as well as with tunicamycin (TM) in liquid cultures (mean ± SD, $n = 3$ independent experiments or representative picture shown); FL-1 to FL-3, fluconazole-induced *petites*; MO-1 to MO-3 macrophage-derived *petites*; *C.g.*, *C. glabrata*.

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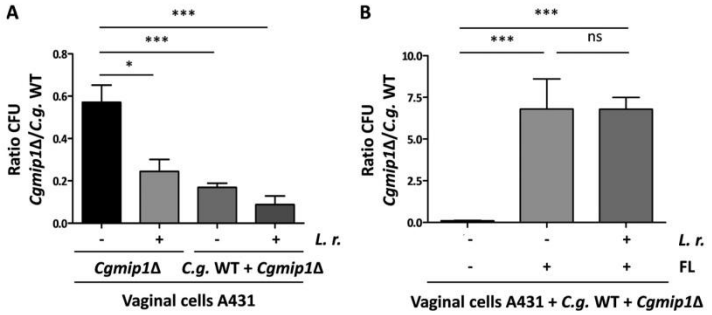

FIG 6 The *Cgmip1Δ petite* phenotype seems to be adaptive under infection-like conditions but not in commensal-like conditions. (A) The ratio of recovered *CgMIP1*-deleted to wild-type *C. glabrata* cells was low when they were incubated separately on vaginal cells (*Cgmip1Δ*/–). The presence of *Lactobacillus rhamnosus* (*L. r.*) further shifts that ratio toward a lower recovery of *Cgmip1Δ*, and these effects are exacerbated in a direct competition in the same wells (*C. glabrata* [*C. g.*] WT + *CgmipΔ*). Data shown are the mean ± SD; $n = 3$ independent experiments. (B) In an infection model in the presence of fluconazole (FL), the effect is inverted. Without fluconazole, *CgmipΔ* is outcompeted as before (note the scale), but it has a decisive advantage in the presence of the antifungal drug, independent of the commensal bacteria (mean ± SD, $n = 3$ independent experiments). Statistically significantly different values (unpaired, two-sided Student's *t* test on log-transformed ratios) are indicated by asterisks as follows: *, $P \leq 0.05$; ***, $P \leq 0.001$.

stressors cannot be solely dependent upon Pdr1-regulated pathways or functions. Evidently, additional resistance mechanisms exist in the *petite* phenotype. In agreement with these results, the double mutant *Cgmip1Δ*+*pdr1Δ* showed significantly higher survival than the wild type after phagocytosis by hMDMs, whereas a *pdr1Δ* single mutant was actually killed more (Fig. S2D). Thus, the Pdr1 pathway seems to be partially involved in stress resistance and macrophage survival of these strains, but this cannot be the sole underlying mechanism.

Overall, these results show that *petite* strains possess a constitutively active detoxifying response that, together with the overexpression of efflux pumps, confers ER stress resistance (in addition to azole resistance) and could explain the higher fitness within the phagosome observed at early time points.

The *Cgmip1Δ petite* phenotype might be adaptive under infection-like conditions but not in commensalism. We propose that the generally reduced growth of *petite* mutants will be disadvantageous when competing with other microorganisms in a commensal environment, such as the human gut or vagina. Therefore, we wondered whether the emergence of *petite* phenotype may only be adaptive under infection-like conditions, such as antifungal treatment with azoles. To test this, we incubated separately or simultaneously the wild type and *Cgmip1Δ* in the presence of vaginal cells and *Lactobacillus rhamnosus* for 24 h, mimicking a commensal-like situation (39), in either n the absence or the presence of 8 μg/ml fluconazole. This fluconazole level is three times the concentration reported in vaginal fluids after a single oral dose (40), but a concentration half lower than the MIC_{50} for *C. glabrata* (34). As expected, we observed only 60% of the wild-type CFU for the *petite* strain when it was incubated alone with human epithelial cells and without antifungals after 24 h (Fig. 6A). The relative growth of *Cgmip1Δ* was further reduced in the presence of lactobacilli, and even more when coincubated with the wild type and bacteria (Fig. 6A). However, the *petite* strain massively out-competed the wild type when fluconazole was present (Fig. 6B). Surprisingly, we did not observe a reduction in the relative *Cgmip1Δ* CFU in the presence of both lactobacilli and fluconazole, as it was seen when both species grew together in the absence of the drug. The increased resistance to fluconazole of *Cgmip1Δ* may provide a better fitness in an acidic environment since it is known that fluconazole and acidic conditions created by *Lactobacillus* have a

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synergistic activity. Indeed, fluconazole is known to be fungicidal in the presence of lactic acid for *C. albicans* (41) and acetate for *C. albicans* and *C. glabrata* (42).

In conclusion, these results show how in commensal-like and nontreated conditions, *Cgmip1*Δ is outcompeted by respiratory-competent yeast cells and is also less able to compete with commensal bacteria. We conclude that the *petite* phenotype likely emerges only under conditions where it is advantageous and then exists only transiently. These conditions include fluconazole treatment, but also uptake by macrophages.

The *Cgmip1*Δ *petite* phenotype is found in clinical strains. Our data so far indicate that the *petite* phenotype should only appear transiently or at low rates in patients but then provide significant advantages by increasing resistance to both phagocytes and antifungals. We therefore screened two collections of 146 clinical *C. glabrata* isolates in total, provided by two different laboratories. For sample collection, the incubation time was specifically extended to detect slower-growing yeasts. Sixteen strains were identified as *petite*; i.e., they showed a small colony size, absence of mitochondrial reductive power (Fig. S3A), and no growth in alternative carbon sources (Fig. S3B). The only common clinical characteristic these strains show is that the majority of them were isolated from patients with other underlying diseases (Table S1). Importantly, many of the *petites* were isolated from patients not currently undergoing antifungal therapy, and none were under azole treatment (Table S1). These strains showed the absence (likely rho^0) and sometimes increased amounts of the mtDNA (rho^-) fragment we screened for (Fig. S3B), without any visible difference in the phenotypes we tested for. Furthermore, like our experimentally created *petites* and independent of the amounts of mtDNA detected, clinical *petites* exhibited high resistance to azoles (Fig. S3C), although they had not been exposed to azole treatment (Table S1), and generally showed a higher survival inside macrophages at early time points compared to the wild type (Fig. S3D). Lastly, they grew better under ER stress than the wild type (Fig. S3E). These results indicate that the *petite* mutant can emerge during *C. glabrata* infections *in vivo* in clinical settings and that these exhibit all the resistances and characteristics we found in the experimentally created *petite* strains.

***CgMIP1* sequencing of *petite* clinical isolates.** The fact that the mitochondrial polymerase gene *CgMIP1* shows a high value of positive selection (d_N/d_S = 3.40) during the diversification of *C. glabrata* as a species and the presence of *petite* strains in clinical isolates of *C. glabrata* led us to hypothesize that these two phenomena are connected. We therefore searched for mutations of *CgMIP1* by obtaining the genome sequences of 14 clinical strains isolated from 7 different patients, of which 13 were *petite* and 1 was respiratory competent. In comparison, we used the reference *C. glabrata* strain ATCC 2001 and 16 respiratory-competent clinical strains, whose genome sequences have been previously obtained (43). Compared to the wild-type strain, we found different mutations along the *MIP1* gene sequence, but we did not observe a specific common mutation pattern for the *petite* strains (Fig. 7). Furthermore, none of these mutations were found in the predicted polymerase or exonuclease domains, which are important for the function of the protein (44). Interestingly, we found variations of the N-terminal mitochondrial targeting sequence, which we determined by TargetP 2.0. Twelve *petites* (98.3%) show insertions of up to three more amino acids in the positions 24S, 25M, and 26L/R, in comparison to the respiratory-competent strains, from which only six contained such insertions (35.3%). Furthermore, we looked for similar mutations in other proteins with known or expected mitochondrial localization. Dss1 is an exonuclease of the mitochondrial degradasome, and *CAGL0K03047g* is an ortholog of the *S. cerevisiae ABF2* gene, which has a role in mtDNA replication. Like Mip1, both are essential for mitochondrial biogenesis (45) and maintenance (46). Hem1 is localized in the mitochondrial matrix and required for heme biosynthesis in *S. cerevisiae*. Pgs1 is a mitochondrially localized protein whose deletion leads to increased azole resistance (26), and Pup1 is a mitochondrial protein that is upregulated by Pdr1 in azole-resistant strains (23). We did not detect variability similar to Mip1's in any of these investigated protein sequences, independent of whether a well-defined mitochondrial transfer peptide was detectable (Abf2, Hem1) or not (Dss1, Pgs1, Pup1).

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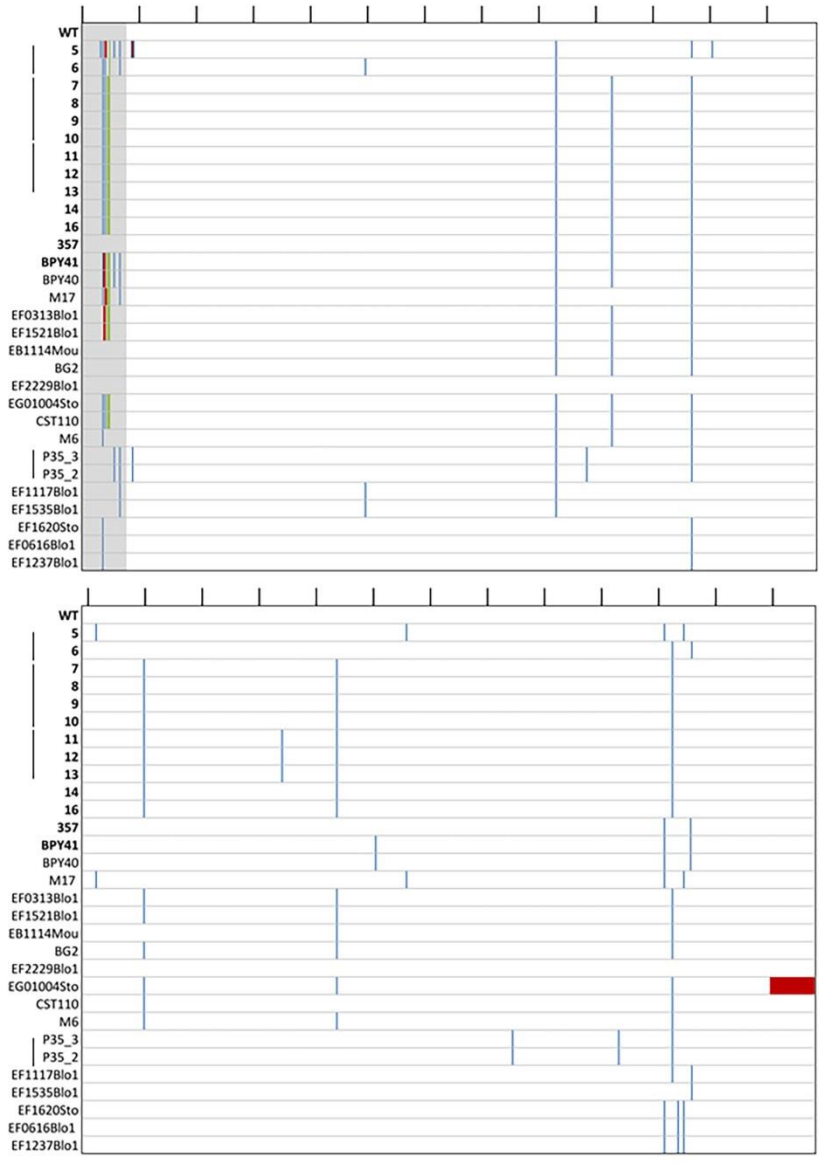

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FIG 7 Mutation on the protein sequence of *CgMIP1* of *C. glabrata* clinical strains compared to the wild type. Wild type (ATCC 2001, WT); the first 14 strains marked in bold are *petite* mutants, and below there are the 17 respiratory-competent strains. Gray, predicted mitochondrial signal peptide of the wild type; blue, amino acid substitutions; green, insertions; red, deletions. The black lines on top indicate the amino acid position every 50 amino acids. The black lines next to the strain names indicate that those strains have been isolated from the same patient.

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DISCUSSION

Despite the fact that *C. glabrata* is phylogenetically more closely related to the baker's yeast *S. cerevisiae* than to the pathogenic *C. albicans*, it has by now become the most important non-*Candida albicans Candida* (NCAC) species to cause disease and is of growing concern in clinics (47). The relatively low host cell damage and immune responses that *C. glabrata* elicits, combined with its ability to survive and replicate within macrophages, indicates that stealth and persistence are its main virulence strategies during infection (4). However, residing for a long time within the human body requires phenotypic plasticity to survive changing stresses, such as osmotic, ER, and oxidative stress, hypoxia, and starvation (48–53). One mechanism that leads to the better host adaptation and virulence in human-pathogenic bacteria and fungi is the inactivation or loss of specific genes, which are then known as antivirulence genes (10, 11).

Specifically, in *C. albicans* and *C. glabrata* mutations, decreasing mitochondrial function can affect host-pathogen interactions. Although *C. albicans* is considered a *petite*-negative species (13), a mutant with uncoupled oxidative phosphorylation was recovered after serial passaging of wild-type *C. albicans* through murine spleens (54). It showed higher tolerance to ROS, altered cell wall composition, resistance to phagocytosis by neutrophils and macrophages, as well as an increased persistence and higher fungal burden in mice. Of note, in contrast to its progenitor, this strain did not kill infected mice any more. The authors pointed out that the uncoupled oxidative phosphorylation lowers intrinsic ROS production, which may be advantageous inside the phagosome, while the altered cell wall composition may diminish immune recognition. The mutant also showed a lower susceptibility to fluconazole and voriconazole due to increased expression of the efflux pump-encoding gene *MDR1* (55). In another example, a respiratory-competent *C. glabrata* strain and its *petite* mutant were sequentially isolated from a patient undergoing long-term fluconazole treatment (22, 23). The mutant showed a high expression of virulence- and efflux pump-related genes, oxido-reductive metabolism and stress response genes, as well as cell wall-related genes. It also led to a higher mortality in neutropenic mice and a higher tissue burden in immunocompetent mice (23), and it was also resistant to azoles due to the *PDR1*-dependent upregulation of the efflux pump-encoding genes *CDR1* and *CDR2*. The mitochondrion-related gene, *PUP1*, was also strongly upregulated in a *PDR1*-dependent manner. Interestingly, enhanced virulence of *C. glabrata* associated with high upregulation of *CDR1* and *PUP1* has been observed in azole resistance clinical isolates resulting from gain of function mutations (GOF) in the *PDR1* gene (23). It was therefore speculated that both genes may contribute to favor *C. glabrata* in host interactions, in a still unknown manner. Thus, the *petite* phenotype may constitute a relevant pathogenic form of *C. glabrata*, and genes involved in mitochondrial function may be considered potential antivirulence genes (11).

In agreement with these previous findings, our study highlights the adaptive advantage that the lack of mitochondrial function has for *C. glabrata* under infection conditions. Likely not coincidentally, the mtDNA polymerase-encoding gene *CgMIP1* seems to have been under selective pressures during the evolution of *C. glabrata* (5). We found that deletion of this gene leads to loss of mtDNA and triggers the *petite* phenotype in both *C. glabrata* and *S. cerevisiae*. This phenotype confers a survival advantage at early time points after phagocytosis, but only for *C. glabrata* and not for *S. cerevisiae*. In addition, *petite* variants appeared from phagocytosed wild-type cells at an appreciable frequency, especially at early time points during their interaction with macrophages. We argue that these were likely induced by the intraphagosomal environment and provided an immediate advantage to the fungus. However, within the phagosome, glucose is absent, and only alternative carbon sources are available (carboxylic acids, amino acids, peptides, *N*-acetylglucosamine, and fatty acids), which require mitochondrial oxido-reductive power for their metabolism (29, 30). We assume that this is the reason that in the long term, after 4 and 7 days, the advantage of the *petite* phenotype is reverted, as they starve and are recovered in ever lower numbers.

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Of note, the fact that in this experimental model oxygen is present at normal atmospheric levels may confer an advantage to the respiratory-competent wild type. In infected tissue *in vivo*, in contrast, oxygen levels are low, and it is known that hypoxia modulates the innate immune response and enhances phagocytosis (56, 57). In this case, the *petite* phenotype may even confer an adaptive advantage over respiratory-competent yeasts, which must rewire their metabolism upon confrontation with phagocytes. In addition, we observed that the *petite* phenotype can reverse if the fungi find themselves outside macrophages and the associated stresses. It seems feasible therefore that macrophage-induced *petites* may regain their normal growth behavior *in vivo* if the fungus escapes the phagocytes and the change into a *petite* phenotype represents a temporary adaptation of *C. glabrata* to adverse conditions.

Importantly, in addition to reversible *petites*, we collected several nonreversible macrophage-derived *petite* variants that allowed us to study their phenotype. They showed high resistance to azoles, in addition to other typical *petite* characteristics. In turn, stable fluconazole-derived *petites* showed the expected loss of susceptibility to azoles and, surprisingly, a better survival to phagocytosis as well. It is known that fluconazole can trigger (temporary) loss of mitochondrial function in *C. glabrata* and *S. cerevisiae* and, as a consequence, an increased fluconazole resistance (16–18, 21). However, to our knowledge this study shows for the first time that phagocytosis and intraphagosomal residence can lead to the emergence of fluconazole resistance, and vice versa, in a potentially clinically important cross-resistance phenomenon.

The mechanistic basis of how these resistances develop is not completely understood: in contrast to our collection of clinical *petites*, the clinical *petite* variants reported so far have all been isolated from patients undergoing fluconazole treatment (19, 22), and most of the *petite* isolates analyzed here lack mtDNA, but synthesis inhibition or degradation of mtDNA by the action of azoles has not yet been reported. In fact, it was shown that azole exposure does not always lead to a loss of mtDNA, but rather, damages mitochondrial components (17, 18, 21). Fluconazole-induced *petite* can revert at a frequency of 1.5×10^{-2} (21), which suggests a genetic or epigenetic regulation. Our data indicate that *C. glabrata* turns *petite* within the macrophages due to phagosomal oxidative stress, since we observed similar conversions during incubation with H_2O_2. It is known that oxidative stress can trigger mitochondrial damage and loss of activity by affecting mitochondrial membrane permeability, the respiratory chain, or the mtDNA (31, 32). These factors could be at work during the induction of the *petite* phenotype in macrophages. Importantly, like azole-induced *petites*, we found that a fraction of these macrophage-derived *petite* phenotypes were reversible.

The loss of mitochondrial function also activates compensatory pathways, such as detoxifying mechanisms and cell wall remodeling (33). Indeed, all our *petite* strains showed constitutive expression of the efflux pump-related (and azole resistance-mediating) genes *PDR1* and *CDR1*, and some transcription of heat shock protein-encoding genes such as *HSP12* and *HS42* and *SGA1* (23, 58). Moreover, all *C. glabrata petites* exhibited a surprisingly high resistance to ER stressors, which was not found in *S. cerevisiae*. We suggest that this ER stress resistance is induced by mitochondrial dysfunction, possibly by way of the interorganelle tether ERMES (endoplasmic reticulum and mitochondrial encounter structures). Interestingly, so far, only the opposite direction has been observed, where ER stress leads to mitochondrial dysfunction (59). Our data may therefore hint toward a bidirectional signaling between the two organelles. This response also seems to confer an adaptive advantage in the phagosome, since it has been shown that ROS may act not alone in killing the fungus but in combination with additional stresses; suppression of ROS by macrophages alone does not increase the fungal survival (6). In addition, this ER stress resistance would also confer protection against the generic ER stress that pathogens face during infection (35, 60, 61). We also showed that the *PDR1* pathway is at least partially involved in the ER stress resistance as well as in macrophage survival, in agreement with previous findings (23). The majority of *petites* also showed increased expression of members of the *YPS* gene family. It

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has been suggested that Yps-mediated cell wall remodeling can play a role in altering or suppressing macrophage activation (35), and we hypothesize that this may contribute to the better survival of *petite* variants within the phagosome. However, the exact mechanisms for the increased survival of *petite* remains unknown, not the least because the general basis for *C. glabrata* macrophage survival is still largely unknown (6). Of note, *S. cerevisiae*, which did not benefit from a *petite* phenotype in macrophages, also did not show constitutive *YPS* orthologue expression or ER stress resistance. Higher oxidative stress resistance does not seem to play a role, as *Cgmip1*Δ grew similarly to the wild type under oxidative stress. In contrast, different mitochondrial mutants of *S. cerevisiae* even show increased sensitivity to oxidative stress (36). The altered cell wall, and especially the increased mannan exposure of *Cgmip1*Δ, can explain the increased phagocytosis rate, as this is known to be mannan-dependent (25, 62), but also a better survival (27). Clearly, the induction of a *petite* phenotype by either azoles or phagocytosis had a strong influence on the macrophage-fungus interactions, but overall, benefits *C. glabrata* in the phagosome.

The *petite* phenotype shows, nonetheless, a strong handicap in fitness and competitiveness in our commensal-like model, likely due to its slow growth, as observed before (63). However, in our model of vaginal candidiasis treated with fluconazole, the *petite* phenotype shows a steady advantage. Therefore, we suggest that the *petite* phenotype, which also appears naturally and in the absence of stress at low frequencies, serves as a bet-hedging strategy to face stressful conditions, such as phagocytosis or azole exposure, in *C. glabrata*. Similar phenotypes are described in bacteria, for which it is known that microorganisms that give rise to heterogeneous populations and phenotypic switching are more likely to survive in fluctuating environments than otherwise "stable" populations (64, 65). Important intracellular pathogens such as *Staphylococcus aureus* and *Salmonella* are known to form small colony variants (SCVs), a phenotype analogous to *petite*, as part of the bacterial heterogeneity that might confer an adaptive advantage upon environmental changes (64, 66, 67). These show a decrease in antibiotic susceptibility and link to chronic and relapsing, often therapy-refractory, infections. Moreover, they show reduced expression of virulence factors and higher adhesion, which promotes internalization in host cells, and facilitate immune-evasion and long-term persistence within their hosts (68, 69). SCVs from many Gram-positive and -negative bacteria have been recovered from clinical tissues (68, 69). On the yeast counterpart, so far, only a *petite* mutant of *C. glabrata* has been reported to possess any pathogenic advantage (23). Analogous to the SCVs, these mutants have been isolated from cases of antifungal treatment (19) and recurrent fungemia (22), with a decreased susceptibility to antifungals and increased fungal burden in animal models of infection (23). Furthermore, recently, it has been shown that a negative correlation between fitness costs derived from drug resistance and virulence is not always the case, but in fact, virulence could be maintained or even increased in the presence of such costs that result in a reduced growth rate in *C. glabrata* (70). This agrees with our hypothesis of the *petite* phenotype as an advantageous strategy during infection despite its slower growth. The same slow growth within macrophages, however, also led to depletion of *petites* in our long-term experiments over a week, making it more difficult to imagine a permanent selection advantage of this phenotype in the human host. Two things, however, must be considered, first that the oxygen level (and with it the relative advantage of functional mitochondria) is lower within tissue than in cell culture or liquid medium growth experiments (71). Second, the *petites* may also escape macrophages before these effects come into play, either via an unknown, potentially nonlytic escape route or by bursting the macrophage (9, 72). A highly interesting possibility in this context is that the presence of cohabiting *petites* may benefit wild-type cells in the same macrophage. In *Cryptococcus gattii*, a spontaneous-appearing variant with tubular mitochondria is itself replication-deficient but resistant to phagosomal stresses (73). In coinfected macrophages, this seems to protect strains with normal mitochondria and increases their proliferation significantly. Whether a similar "division of

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labor," as the authors call it (73), may allow *petites* to help wild-type *C. glabrata* (and potentially benefit themselves by having the wild type burst the macrophage due to their growth) is an interesting open question that we will address in the future.

Given the potential advantages of *petite* variants during infection, one would expect to frequently find *C. glabrata petite* phenotypes in clinical samples, but although *petites* have been reported (19, 22), these reports seem to be rare. Nonetheless, when we specifically looked for the *petite* phenotype in clinical isolates, we found 16 of these strains even in the absence of antifungal treatment. They shared all the hallmark features with *Cgmip1*Δ, macrophage-, and fluconazole-derived *petites*; the majority showed loss of mtDNA, azole resistance, increased survival upon phagocytosis, and increased ER stress profile, which suggests a common mode of resistance within the macrophages. It may well be that *C. glabrata petite* phenotypes—possibly triggered by interactions with macrophages—are actually more common in clinical samples but potentially overlooked because of their slow growth.

We started these investigations because of the signs of recent selection on the mitochondrial DNA polymerase gene *CgMIP1*. We hypothesized that this could indicate a role in the adaptation of *C. glabrata* to host environments. Indeed, some (but not all) of the *petite* clinical isolates we investigated showed mutations in the *CgMIP1* gene sequence, and the majority showed an insertion pattern in the presumable mitochondrial targeting sequence, which may relate to their *petite* phenotypes via a reduction of mitochondrial polymerase function. In addition, we found that other mitochondrion-related proteins did not show similar mutation frequency, which supports the idea that CgMip1 may still be a target for selection. Moreover, it has been shown that point mutations in the polymerase domain of the orthologous *MIP1* gene in *S. cerevisiae* can trigger emergence of *petites*, and this frequency increased with higher temperatures. Specifically, the mutation E900G yielded from 6% *petites* at 28°C to 92% at 36°C (74). While many of these *petites* were rho^0—completely devoid of mtDNA—some still contained amplified mtDNA fragments that map to various positions of the mtDNA and were considered rho$^-$ (74). These mtDNA fragments can rescue strains that are respiratory deficient due to mutations in mtDNA by recombination after crossing (75, 76). Interestingly, we observed mtDNA fragments in our sequence data of clinical *petite* strains (data not shown), similar to *S. cerevisiae* rho$^-$. We cannot discard the possibility that mtDNA can be restored by recombination after crossing *in vivo*, since while it has not yet been observed, *C. glabrata* has most likely not lost its ability to mate (43, 77). In addition, we also found that the wild-type strain of *C. glabrata* and its two most closely related human pathogens, *Nakaseomyces nivariensis* and *Nakaseomyces bracarensis*, show the substitution E926D in Mip1 compared to the environmental species *Nakaseomyces delphensis*, *Nakaseomyces bacillisporus*, and *Candida castelli*. Since this is equivalent to the E900 position in *S. cerevisiae*, which upon mutation increases the frequency of *petite* occurrences, we hypothesize that, on the one hand, *C. glabrata* could more easily turn *petite* than environmental *Nakaseomyces* species, but on the other hand, it can also retain mtDNA in its genome as a possible mechanism to recover mitochondrial function once the *petite* phenotype is not adaptive. These tantalizing hypotheses will be tested in the near future.

Alternatively, the *C. glabrata CgMIP1* gene sequence may be the target of epigenetic regulation, as epigenetics have been suggested to be involved in the reversion of *petite* phenotype to wild type (21). In these models, mtDNA levels are reduced down to a (near) complete loss of mitochondrial function. Upon resumption of polymerase function, the mitochondrial function and growth then revert to normal. The ability to switch between *petite* and non-*petite* phenotype would confer *C. glabrata* an important phenotypic plasticity to adapt to the host's changing environments; the *petite* phenotype may be less competitive as a commensal but much fitter in infection situations with active phagocytes and antifungal treatment.

In conclusion, this study shows how temporary mitochondrial dysfunction triggers a *petite* phenotype in *C. glabrata* under infection-like conditions, with the potential to

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confer cross-resistance between the macrophages and azole antifungal treatment. This has three implications. First, it adds mitochondrial function to the list of potential antivirulence factors, since its loss results in a gain in virulence potential in the form of stress resistance. Second, it has implications for the treatment of *C. glabrata*, as fluconazole may inadvertently increase the fitness of the fungus against the innate host defenses. Due to the *petite* morphology and long generation times, such resistant isolates may then be missed in standard diagnostics. Third, our observations provide a potential clinically relevant route for the emergence of azole resistance of *C. glabrata* by immune activities, a new paradigm in the development of antifungal resistance.

MATERIALS AND METHODS

Screening and acquisition of suspected petite *C. glabrata* strains. In the course of routine diagnostics—executed by the Institute of Hygiene and Microbiology in Würzburg, Germany—all accumulated chromogenic *Candida* agar plates (CHROMagar; Becton, Dickinson, New Jersey, USA) were systematically collected and incubated for at least 7 days at 37°C prior to screening. After incubation, plates were visually screened concerning growth, color, and morphology. Only suspected *C. glabrata* small colony variants were subisolated and reincubated for a minimum of 3 days at 37°C. In case of a confirmed growth behavior, final species identification was executed by matrix-assisted laser desorption ionization–time of flight mass spectrometry MALDI-TOF) (bioMérieux, Paris, France).

From a total of 3,756 agar plates—originating from various clinical specimens examined between November 2019 and June 2020—466 exhibited growth after incubation. A total of 525 different strains were identified through chromogenic media, of which the majority (312) presented in a green color, suggesting *C. albicans*, whereas in 170 cases mauve colonies were observed. A total of 82 of these were identified as *C. glabrata* using MALDI-TOF. Based on morphology, 41 strains were suspected to be *petites*, which was finally confirmed in 20 cases.

Strains and growth conditions. All strains used in this study are listed in Table S2. The *C. glabrata* mutant strains are derivatives of the laboratory strain ATCC 2001. In each strain a single open reading frame (ORF) was replaced with a bar-coded NAT1 resistance cassette in the strain ATCC 2001. All yeast strains were routinely grown overnight in YPD (1% yeast extract, 1% peptone, 2% glucose) at 37°C in a 180-rpm shaking incubator.

To analyze sensitivity to H_2O_2 and ER stress, 5 μl of serial diluted yeast cultures (10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 cells/ml) were dropped on solid YPD medium (YPD, agarose 2%) containing increasing concentrations of H_2O_2 (7.5 mM, 10 mM, and 12 mM), tunicamycin (2 μg/ml), DTT (10 mM) or 2-mercaptoethanol (12 mM). Pictures were taken after 48 h of incubation at 37°C, unless indicated otherwise.

Mitochondrial activity was visualized by growing serial dilutions of the strains on YPD agar containing 0.02% TTC (2,3,5-triphenyltetrazolium chloride) (Sigma-Aldrich) and incubating cells in minimum medium (1% yeast nitrogen base, 1% amino acids, 0.5% ammonium sulfate) with 4% glycerol as the sole carbon source for 4 days at 37°C in a 180-rpm shaking incubator.

Growth assays. To analyze stress sensitivity, 5 μl of a yeast cell suspension (2×10^7 cells/ml) was added to 195 μl medium in a 96-well plate (tissue culture test plate; TPP Techno Plastic Products AG) containing liquid YPD or YPD and different concentrations of fluconazole (10, 25, 35, 50, 75, and 100 μg/ml) and tunicamycin (0.5, 1, 1.5, 2, and 3 μg/ml). For the ER stress analysis, yeasts were incubated in YPD and tunicamycin (1.5 μg/ml). The growth was monitored by measuring the absorbance at 600 nm every 30 min for 150 cycles at 37°C, using a Tecan reader (Infinite M200 PRO plate reader; Tecan Group GmbH) with orbital shaking. All experiments were done in independent biological triplicates on different days and are shown as the mean with standard deviation (SD) for each time point.

The MIC_{50} was determined in a 96-well plate (tissue culture test plate; TPP Techno Plastic Products AG) containing minimum medium (1% yeast nitrogen base, 1% amino acids, 0.5% ammonium sulfate, and 2% glucose) with increasing concentrations of fluconazole (FL) (4, 8, 16, 32, 64, 128, and 256 μg/ml), voriconazole (VC) (0.5, 1, 2, 4, 8, and 16 μg/ml), and clotrimazole (CL) (1, 2, 4, 8, and 16 μg/ml) at 37°C. AQ: B

Fungal RNA isolation. For preparation of RNA from *in vitro*-cultured *Candida* cells, stationary-phase yeast cells were washed in phosphate-buffered saline (PBS) and the optical density (OD) was adjusted to 0.4 in 5 ml liquid YPD. After 3 h, cells were harvested and centrifuged. The isolation of the fungal RNA was performed as previously described (78). The RNA was then precipitated by adding 1 volume of isopropyl alcohol and one-tenth volume of sodium acetate (pH 5.5). The quantity of the RNA was determined using the NanoDrop spectrophotometer ND-1000 (NRW International GmbH).

Expression analysis by reverse transcription-quantitative PCR (qRT-PCR). The cDNA was synthesized from DNase-treated RNA (1,000 ng) using 0.5 μg oligo-$dT_{12\text{-}18}$, 100 U Superscript III reverse transcriptase, and 20 U RNaseOUT recombinant RNase inhibitor (all from Thermo Fischer Scientific) in a total volume of 20 μl for 2 h at 42°C followed by heat inactivation for 15 min at 70°C. Quantitative PCR with EvaGreen QPCR mix II (Bio&SELL) was performed with 1:10 diluted cDNA. Primers were used at a final concentration of 500 nM. Target gene expression was calculated using the $\Delta\Delta CT$ method (79), with normalization to the housekeeping genes *CgACT1* for *C. glabrata* or *ScACT1* for *S. cerevisiae*. For mtDNA quantification, yeast DNA was extracted following the Harju et al. protocol (80), and 100 ng was the reaction concentration. *ScCOX3* and *CgCOX3* were used as mitochondrial target genes and *CgACT1* or *ScACT1* as housekeeping genes. All experiments were done in independent biological triplicates on different days and are shown as the mean with standard deviation (SD) for each time point. AQ: C

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Chitin, mannan, and β-glucan exposure. To measure chitin content, yeasts from an overnight culture were washed in PBS and incubated with 9 ng/ml of wheat germ agglutinin (WGA)-FITC diluted in PBS for 1 h at room temperature. After washing with PBS, fluorescence was quantified by flow cytometry (BD FACS Verse; BD Biosciences, Franklin Lakes, NJ, USA) counting 10,000 events. For mannan quantification, yeast cells were washed with PBS and incubated with concanavalin A-647 for 30 min at 37°C. After washing with PBS, fluorescence was quantified also by flow cytometry. For β-glucan staining, yeast cells were washed with PBS and incubated with 2% bovine serum albumin (BSA) for 30 min at 37°C, followed by a first step of 1 h of incubation with a monoclonal anti-β-glucan antibody (Biosupplies) (diluted 1:400 in 2% BSA) and a second step of 1 h of incubation with an Alexa Fluor 488 conjugate secondary antibody (Molecular Probes) (diluted 1:1,000 in 2% BSA). Fluorescence was again quantified by flow cytometry. All experiments were done in independent biological triplicates on different days, and shown as the mean with standard deviation (SD) for each time point.

Isolation and differentiation of human monocyte-derived macrophages (hMDMs). Blood was obtained from healthy human volunteers with written informed consent according to the declaration of Helsinki. The blood donation protocol and use of blood for this study were approved by the Jena institutional ethics committee (Ethik-Kommission des Universitätsklinikums Jena, permission no. 2207-01/08). Human peripheral blood mononuclear cells (PBMCs) from buffy coats donated by healthy volunteers were separated through lymphocyte separation medium (Capricorn Scientific) in Leucosep tubes (Greiner Bio-One) by density centrifugation. Magnetically labeled CD14-positive monocytes were selected by automated cell sorting (autoMACs; MiltenyiBiotec). To differentiate PBMC into human monocyte-derived macrophages (hMDMs), 1.7×10^7 cells were seeded into 175-cm^2 cell culture flasks in RPMI 1640 medium with L-glutamine (Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS; Bio&SELL) and 50 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF; ImmunoTools). Cells were incubated for 5 days at 37°C and 5% CO_2 until the medium was exchanged. After another 2 days, adherent hMDMs were detached with 50 mM EDTA in PBS and seeded in 96-well plates (4×10^4 hMDMs/well) for the survival assay, in 12-well-plates (4×10^5 hMDMs/well) for the intracellular replication assay with 100 U/ml interferon-γ (IFN-γ), and in 24-well-plates for the long-term experiment (1.5×10^5 hMDMs/well) without IFN-γ. Prior to macrophage infection, medium was exchanged with serum free-RPMI medium and 100 U/ml IFN-γ. For the long-term experiment, medium was exchanged with RPMI 1640 containing 10% human serum (Bio&Sell 1; B&S human serum sterilized AB male, lot BS.15472.5).

AQ: D

Phagocytosis survival assay. Mutant strains were washed in phosphate-buffered saline (PBS), and total numbers of cells were assessed by the use of a hemocytometer. MDMs in 96-well-plates were infected at a multiplicity of infection (MOI) of 1, and after 3 h and 6 h of coincubation at 37°C and 5% CO_2, non-cell-associated yeasts were removed by washing with RPMI 1640. To measure yeast survival in MDMs, lysates of infected MDMs were plated on YPD plates to determine CFU.

The long-term experiment was performed in 24-well-plates where the cells were infected with an MOI of 1 and incubated for 1 week at 37°C and 5% CO_2. After 3 h of coincubation, cells were washed with PBS, and medium was exchanged with RPMI 1640 containing 10% human serum. At the 3 h time point, both supernatant and lysate were plated. Until the 1-day and 7-day times points, one-third of the medium was exchanged every day with RPMI 1640 with 10% human serum. Then, only the lysate was plated. The lysate of 4 different wells was diluted accordingly, and 200 CFU were plated on 4 YPD agar plates, which were then incubated for 48 h at 37°C. The frequency of *petites* was determined via small colonies that were unable to grow with 4% glycerol as the sole carbon source in minimal medium (1% yeast nitrogen base, 1% amino acids, 0.5% ammonium sulfate). The growth was assayed for 3 days at 37°C in a 180-rpm shaking incubator. The frequency of spontaneous *petites* was calculated by incubation of 1.5×10^5 cells ml^{-1} in RPMI 1640 for 7 days. At 3 h, 1 day, 4 days, and 7 days samples were collected and diluted, and 200 CFU were plated on 6 YPD agar plates, which were incubated for 48 h at 37°C.

Replication within hMDMs. To quantify yeast intracellular replication, *C. glabrata* cells were labeled with 0.2 mg/ml fluorescein isothiocyanate (FITC) (Sigma-Aldrich) in carbonate buffer (0.15 M NaCl, 0.1 M Na_2CO_3, pH 9.0) for 30 min at 37°C. Then, yeast cells were washed in PBS, and macrophages were infected at an MOI 5 for 6 h. Afterward, macrophages were washed with PBS and lysed with 0.5% Triton-X-100 for 15 min. Released yeast cells were washed with PBS, with 2% BSA in PBS, and counterstained with 50 μg/ml Alexa Fluor 647-conjugated concanavalin A (ConA) (Molecular Probes) in PBS at 37°C for 30 min. The ConA-AF647-stained yeast cells were washed with PBS and fixed with Histofix (Roth) for 15 min at 37°C. As FITC is not transferred to daughter cells, differentiation of mother and daughter cells was possible; the ratio of FITC-positive and -negative yeast cells was evaluated by flow cytometry (BD FACS Verse; BD Biosciences, Franklin Lakes, NJ, USA), counting 10,000 events. Data analysis was performed using the FlowJo 10.2 software (FlowJo LLC, Ashland, OR, USA). The gating strategy was based on the detection of single and ConA-positive cells and exclusion of cellular debris.

For quantification of intracellular replication by fluorescence microscopy, cells were fixed with Histofix (Roth) after incubation with macrophages and stained for 30 min at 37°C with 25 μg/ml ConA-AF647 (Molecular Probes) to visualize nonphagocytosed yeast cells. Then they were mounted cell side down in ProLong Gold antifade reagent (Molecular Probes). As FITC is not transferred to daughter cells, differentiation of mother and daughter cells was possible, and intracellular replication was observed by fluorescence microscopy (Leica DM5500B and Leica DFC360).

Detection of ROS in hMDMs. ROS production was measured by luminol-enhanced chemiluminescence quantification, and all cells and reagents were prepared in RPMI 1640 without phenol red. MDMs were grown in white 96-well plates in 100 μl medium per well. Overnight yeast cultures were washed in RPMI 1640 and counted, and 50 μl was added to hMDMs (giving an MOI of 10). For control experiments, hMDMs were left untreated in 150 μl RPMI 1640, or 100 nM phorbol myristate actetate (PMA) (Sigma-Aldrich) was

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added to 50 μl RPMI 1640. All samples were prepared in triplicate. Prior to quantification, 50 μl of a mixture containing 200 μM luminol and 16 U horseradish peroxidase in RPMI 1640 was immediately added. Luminescence was measured every 3 min over a 3-h period of incubation at 37°C using a microplate reader (Tecan Infinite 200).

Macrophage damage assay. Macrophages were infected with *Candida* cells as described above and incubated for 24 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured as a marker for necrotic cell damage using a cytotoxicity detection kit (Roche) according to the manufacturer's instructions. The background LDH value of uninfected macrophages (hMDMs) was subtracted, and the corrected LDH release was expressed as the percentage of high (full lysis) control (maximum LDH release induced by the addition of 0.25% Triton X-100 to uninfected macrophages for 5 min).

Competition assay. This experiment was adapted from Graf et al. (39). A431 vaginal epithelial cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ] no. ACC 91) were routinely cultivated in RPMI 1640 medium with L-glutamine (Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS; Bio&SELL) at 37°C and 5% CO_2 for no longer than 15 passages. For detachment, cells were treated with Accutase (Gibco, Thermo Fisher Scientific). For use in experiments, the cell numbers were determined using a Neubauer chamber system and seeded in a 6-well-plate (4×10^5 cells/well) for 3 days. For infection experiments, the medium was exchanged with fresh RPMI 1640 without FBS. *L. rhamnosus* (ATCC 7469) was grown in MRS broth for 72 h at 37°C. Before infection, bacteria were harvested, washed with PBS, and adjusted to an optical density at 600 nm (OD_{600}) of 0.2 ($\sim 1 \times 108$ CFU/ml) in RPMI 1640. Then, one-third of the total volume of the well of a 6-well-plate was inoculated for 18 h prior to infection with *C. glabrata*. These wells were colonized with *C. glabrata* wild type and mutant separated or mixed in equal cell numbers to a final MOI of 1 for 24 h. The same settings were established in the absence of bacteria as controls. Additionally, in some wells infected with both strains in the presence or absence of bacteria, a final concentration of 8 ng/ml of fluconazole was added. Fluconazole was dissolved in DMSO, and it was ensured that the final percentage of the organic dissolvent in the wells was below 0.1%.

After 24 h, supernatants and attached cells were collected and vortexed thoroughly. Vaginal cells were treated with 0.5% Triton-X-100 for 5 min to lyse them and release adherent fungal cells. Samples were diluted appropriately with PBS. The diluted samples were plated on YPD plates with 1× PenStrep (Gibco, Thermo Fisher Scientific) and incubated at 37°C for 1 to 2 days until adequate growth for determining the CFU was reached.

Sequencing. For the DNA extraction, the strains were grown in YPD cultures for 16 h at 37°C and 180 rpm, and the following protocol was implemented to isolate DNA of high quality. The cultures were centrifuged for 5 min at 4,000 rpm. The pellet was suspended in sorbitol 1 M and centrifuged for 2 min at 13,000 rpm. Then the pellet was resuspended in SCEM buffer (1 M sorbitol, 100 mM Na-citrate pH 5.8, 50 mM EDTA pH 8, 2% β-mercaptoethanol, and 500 units/ml lyticase [MERCK]) and incubated at 37°C for 2 h. Afterward, the samples were centrifuged for 5 min at 13,000 rpm, and the pellet was resuspended in proteinase buffer (10 mM Tris-CL pH 7.5, 50 mM EDTA pH 7.5, 0.5% SDS, and 1 mg/ml proteinase K) and incubated at 60°C for 30 min. Phenol:chloroform:isoamylalkol 25:24:1 was added after the incubation, and the samples were vortexed for 4 min. Then they were centrifuged for 4 min at 13,000 rpm. Then the aqueous phase was transferred to a new tube and a 1:1 volume of cold isopropanol was added. Samples were centrifuged for 15 min at 13,000 rpm. The pellets were washed with 70% ethanol once and centrifuged again for 3 min at 13,000 rpm. After drying, the pellet was resuspended in water and RNase. The genomic DNA was stored at −20°C until sequencing. The sequencing of the clinical strains was done by the company GENEWIZ, using Illumina NovaSeq 2 × 150-bp sequencing and 10 M raw paired-end reads per sample package. Additionally, paired-end reads for non-*petite* clinical isolates were obtained from a previous study (NCBI SRA project SRP099102 [43]). All reads were aligned to the *C. glabrata* reference genome (version s03-mo1-r06 [12]) using Bowtie2 version 2.4.1. Variants were called from the resulting alignments using the call variants script in bbmap version 38.44 (SOURCEFORGE, 2014) with standard parameters. The resulting variant files were applied to the reference genome by the bcftools consensus function version 1.10.2 (81).

***In silico* analysis and statistics.** All the results were obtained from at least three biological replicates (indicated in the figure legends). The means and standard deviations of these replicates are shown. Experiments performed with MDMs were isolated from at least three different donors (see figure legends). Data were analyzed using Prism 5 (GraphPad Software, San Diego, CA, USA). The data were generally analyzed using a two-tailed, unpaired Student's *t* test for intergroup comparisons, if not indicated otherwise.

Data availability. The raw sequencing data that support the findings of this study are available in the Sequence Read Archive (SRA) of the NCBI under the accession number PRJNA665484.

AQ: E

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 0.6 MB.

FIG S2, TIF file, 1.4 MB.

FIG S3, TIF file, 2.4 MB.

FIG S4, TIF file, 2.2 MB.

TABLE S1, DOCX file, 0.04 MB.

TABLE S2, DOCX file, 0.02 MB.

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ACKNOWLEDGMENTS

We thank Daniel Fischer, Marcel Sprenger, Franziska Pieper, Sophie Austermeier, and Stephanie Wisgott for their help and support during isolation of mBMDMs and during isolation and cultivation of hMDMs. Further, we thank Franziska Pieper for her technical assistance with MIC_{50} analysis; Marina Pekmezovic and Marisa Valentine for their help with epithelial cell culture; Volha Skrahina for her technical assistance in creating the double mutant; Marina Pekmezovic for her help with microscopy imaging. We also thank Dominique Sanglard for providing clinical strains of BPY40/41 and Grit Walther for providing clinical strains from the National Reference Center for Invasive Fungal Infections (NRZMyk). The auto-MACS system for magnetic isolation of human monocytes was provided by the research group Fungal Septomics.

This project was supported by the Excellence Graduate School Jena School for Microbial Communication (JSMC) and the Balance of the Microverse Cluster (Germany's Excellence Strategy—EXC 2051—project ID 390713860) at the Friedrich Schiller University of Jena.

We declare no competing interests.

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6.4 Manuscript IV

The involvement of the *Candida glabrata* trehalase enzymes in stress resistance and gut colonization

Mieke Van Ende, Bea Timmermans, Giel Vanreppelen, **Sofía Siscar-Lewin**, Daniel Fischer, Stefanie Wijnants, Celia Lobo Romero, Saleh Yazdani, Ona Rogiers, Liesbeth Demuyser, Griet Van Zeebroeck, Yuke Cen, Karl Kuchler, Sascha Brunke, Patrick Van Dijck

Virulence. 2021; 12(1): 329-345. Published online 2021 Jan 12. DOI: 10.1080/21505594.2020.1868825

Summary:

Candida glabrata is an opportunistic human fungal pathogen and the second most important cause of candidiasis. This fungus uses the sugar trehalose for stress tolerance and also as an energy source. In this article three trehalase enzymes of this pathogen are identified: Ath1, Nth1, and Nth2. The possible role of these enzymes in pathogenicity is characterized by creating single, double, and triple mutants lacking the corresponding genes and subjecting them to *in vitro* and *in vivo* experiments. Ath1 was found to be essential for growth on trehalose as a carbon source; Nth1 was important for oxidative stress resistance, which was verified by the lower survival rate of the *NTH1* deletion strain in human macrophages. No significant phenotype was observed for mutants lacking Nth2. Nevertheless, the triple deletion strain showed a reduced adhesion to catheters, was unable to establish a stable colonization of the gastrointestinal tract in mice, and showed reduced survival in macrophages after four days of phagocytosis. Furthermore, mice infected intravenously with the triple deletion strain appeared healthier than the wild type control. These results shed light on the important role of the trehalose metabolism in *C. glabrata* pathogenicity and open the possibility of new antifungal targets to combat fungal infections caused by this pathogen.

The candidate is:

☐ First author ☐ Second author ☐ Corresponding author ☒ Coauthor

Estimated authors' contributions:

Author	Conception	Data analysis	Experimental	Writing	Provision of the material
MVE	30%	40%	35%	35%	
BT	30%	40%	35%	35%	
GV		10%	10%		
SSL		5%	5%	2%	
DF			2%		
SW			5%		
CLR			1%		
SY			6%		
OR			1%		
LD				3%	
GVZ		2.5%		3%	
YC	10%				
KK					5%
SB	5%	2.5%		2%	20%
PVD	25%			20%	75%

VIRULENCE
2021, VOL. 12, NO. 1, 329–345
https://doi.org/10.1080/21505594.2020.1868825

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RESEARCH PAPER

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The involvement of the *Candida glabrata* trehalase enzymes in stress resistance and gut colonization

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ABSTRACT

Candida glabrata is an opportunistic human fungal pathogen and is frequently present in the human microbiome. It has a high relative resistance to environmental stresses and several antifungal drugs. An important component involved in microbial stress tolerance is trehalose. In this work, we characterized the three *C. glabrata* trehalase enzymes Ath1, Nth1 and Nth2. Single, double and triple deletion strains were constructed and characterized both *in vitro* and *in vivo* to determine the role of these enzymes in virulence. Ath1 was found to be located in the periplasm and was essential for growth on trehalose as sole carbon source, while Nth1 on the other hand was important for oxidative stress resistance, an observation which was consistent by the lower survival rate of the *NTH1* deletion strain in human macrophages. No significant phenotype was observed for Nth2. The triple deletion strain was unable to establish a stable colonization of the gastrointestinal (GI) tract in mice indicating the importance of having trehalase activity for colonization in the gut.

ARTICLE HISTORY
Received 5 September 2020
Revised 28 November 2020
Accepted 17 December 2020

KEYWORDS
Candida glabrata; trehalase; trehalose; colonization; stress; virulence

Introduction

Annually, invasive fungal infections cause 1.5 million deaths [1,2]. *Candida* species are among the most frequently isolated human fungi, with a mortality rate of up to 50% [1,3,4]. Of all *Candida* spp., *Candida albicans* is isolated most frequently [5]. However, the introduction of fluconazole as the first-line antifungal used in the clinic caused a decrease in the number of *C. albicans* infections and introduced an increase in infections caused by inherently more fluconazole tolerant species, such as *Candida glabrata* [5–8]. *C. glabrata* bloodstream infections are associated with high mortality rates and therefore, this fungus is becoming a major threat in hospitals [9,10]. The most important virulence factors of *C. glabrata* are its capacity to grow very rapidly at 37°C, adherence to various substrates and subsequent biofilm formation, its ability to intrinsically tolerate certain antifungal drugs, and its rapid adaptation to stresses [11–13]. In the human body, environmental stresses are ubiquitous, such as nutrient limitation and stress imposed by the host immune response. This activates the *C. glabrata* general stress response, which includes the induction of genes encoding enzymes involved in trehalose metabolism [14].

Trehalose is a non-reducing glucose disaccharide with a double role in fungi. It serves as a storage carbon source but is also crucial as a stress protectant molecule by stabilizing proteins and membranes, thereby causing resistance to antifungal drugs, oxidative stress, and heat stress [15–21]. The trehalose biosynthesis pathway is defined by two main enzymes: trehalose-6-phosphate synthase (Tps1) and trehalose-6-phosphate phosphatase (Tps2). Tps1 converts uridine diphosphate (UDP)-glucose and glucose-6-phosphate into trehalose-6-phosphate (T6P) and UDP, after which Tps2 hydrolyzes T6P into trehalose and free phosphate (Fig S1). Trehalose as well as the enzymes linked to its biosynthesis have been described to be involved in virulence and pathogenesis, with functions in infection, biofilm formation, etc [21–28]. Despite their absence in the human body and their long-known role in virulence, still no Tps1 or Tps2 inhibitor has been discovered and

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Supplemental data for this article can be accessed here.

brought to clinical trials or to the clinic. As trehalose accumulates under stress conditions and thereby "freezes" proteins and membranes, a fast hydrolysis of trehalose upon relief of stress is essential to resume growth in *Saccharomyces cerevisiae* [29,30]. This hydrolysis is mediated by trehalase enzymes. Fungal species generally have two trehalase enzymes: a neutral and an acid trehalase enzyme. In the yeast model organism *S. cerevisiae*, the neutral trehalase enzyme *Sc*Nth1 is regulated by cAMP-dependent phosphorylation and ensures endogenous trehalose metabolization [31–33]. It requires divalent cations (Ca^{2+} and Mn^{2+}) and has a pH optimum of 7 [34,35]. This neutral trehalase shows higher activity during exponential growth on fermentable carbon sources and is located in the cytoplasm of the cells [36]. On the other hand, fungal acid trehalase enzymes contain either a transmembrane domain or a signal peptide and are therefore located on the outside of the fungal cell; in the cell membrane, periplasm or cell wall [37–39]. The extracellular localization ensures hydrolysis of external trehalose [39–41]. In *S. cerevisiae*, the acid trehalase enzyme is also present in the vacuole [37,38]. The acid trehalase enzyme has an optimal pH of 4.5 and is not regulated by cAMP, phosphorylation or divalent cations [36].

Based on sequence homology with the *S. cerevisiae* trehalase enzymes, the *C. glabrata* genome encodes three trehalase enzymes: Ath1, Nth1, and Nth2. *C. glabrata* very rapidly hydrolyzes extracellular trehalose, a feature which is used in the clinic to diagnose *C. glabrata* infections in a quick and cost-effective way [42–48]. Recently, Ath1 (CAGL0K05137g) was found to be responsible for this extracellular trehalose fermentation [41]. The Ath1 orthologues in *C. albicans* (*CaATC1)* and *C. parapsilosis* (*CpATC1)* were found to be involved in virulence and stress resistance. The *CaATC1* and *CpATC1* deletion strains showed both an increased thermotolerance and an increased resistance toward oxidative stress [49–51]. Whereas these results would suggest an increase in virulence, this was not observed using a systemic mouse model, where these deletion strains showed a reduced virulence [49,51]. The *C. glabrata* neutral trehalases, Nth1 (CAGL0M10439g) and Nth2 (CAGL0C04323g) are considered to be cytosolic trehalases responsible for the hydrolysis of intracellular trehalose. In *C. albicans*, deletion of the neutral trehalase *CaNTC1* does not affect virulence in a mouse model of systemic infection [52].

Based on the importance of the trehalose metabolism for virulence in other pathogenic fungi and the observation that *C. glabrata* consumes extracellular trehalose very rapidly, we aimed to characterize the *C. glabrata* trehalase enzymes [49–51]. The trehalase deletion strains were first tested for *in vitro* growth on different relevant fermentable carbon sources. We confirm the data of Zilli, Lopes [41], where it was shown that Ath1 is required for utilization of exogenous trehalose. Moreover, we show that Ath1 is present in the periplasm. Hence, we hypothesized that the Ath1 enzyme could play a role in colonization of the human gut. We tested this in the gastrointestinal tract of mice observing the *C. glabrata* colonization over time. Unexpectedly, the *ath1*Δ mutant showed a wild type phenotype, whereas the triple deletion strain was unable to form a stable colonization of the GI tract. We also showed that *C. glabrata* Nth1 is involved in the oxidative stress response. As *C. glabrata* has the capability to survive and replicate in immune cells, the trehalase mutant strains were tested for survival in human macrophages. After four days, the single deletion strains and the triple deletion strain showed a reduced survival in the macrophages compared to the wild type. Finally, we tested the trehalase deletion strains in a mouse model of systemic infection. Only minor differences were observed between the different strains with a small increase in mortality rate for the *nth1Δ* strain. Together, these results indicate that the trehalase enzymes could be a good target for the development of new antifungal drugs, especially for their colonization in the gut. As we expect that targeting of one trehalase is insufficient, we suggest to target all three trehalase enzymes with a competitive inhibitor.

Materials and methods

Construction of phylogenetic tree

To create the trehalase phylogenetic tree, protein sequences were retrieved from the *Candida* Genome Database (CGD), *Saccharomyces* Genome Database (SGD), *Aspergillus* Genome Database (AspGD), UniProt, and PomBase [53–57]. In CLC Main Workbench, protein sequences were aligned and the phylogenetic tree was constructed using the unweighted pair method with arithmetic mean (UPGMA) and the Kimura Protein as a protein distance measure [58].

Yeast strains, plasmids, primers and media

The yeast strains, plasmids and primers used in this study are listed in Supplementary Table 1. Yeast cells were grown in either YP (1% yeast extract, 2% bacteriological peptone), synthetic complete (SC) medium (1.7 g/L Difco yeast nitrogen base without amino acids and without ammonium sulfate, 0.79 g/L complete supplement mixture [CSM; MP Biomedicals], 5 g/L ammonium sulfate, pH 5.5 (liquid) or 6.5 (solid)), YNB (1.7 g/L Difco yeast nitrogen base without amino acids and without ammonium sulfate, 20 mg/L

tryptophan, 20 mg/L histidine, 30 mg/L leucine, 5 g/L ammonium sulfate, pH 5.5 (liquid) or 6.5 (solid)) or RPMI 1640 medium. These media were supplemented with glucose or trehalose as indicated in the experiment. For solid media, 15 g/L Difco agar granulated was added.

Construction of plasmids

For expression of the flippase enzyme, the nourseothricin cassette of vector pLS9 was replaced by the hygromycin marker resulting in pLS10. The hygromycin marker was amplified using primers C6240 and C6241 from plasmid pgRNA-uni-hph (p58) and subsequently ligated into the *Not*I digested pLS9. Colonies were checked using primers 7883 and B3220 and verified by sequencing. For localization experiments, the trehalase enzymes were fused to mCherry. Therefore, open reading frames (ORFs) were amplified using primers D84 and D1728 (*ATH1*), D88 and D89 (*NTH1*), or D92 and D93 (*NTH2*) from wild type genomic DNA. The terminators were amplified using primers D86 and D87 (*ATH1*), D90 and D91 (*NTH1*), or D94 and D95 (*NTH2)* from wild type genomic DNA. The corresponding ORF and terminator fragments were inserted in *BamH*I and *Xho*I digested pYC56 vector using NEBuilder (New England Biolabs), resulting in the expression vectors pBM13 (*ATH1*), pBM14 (*NTH1*), and pBM15 (*NTH2*). Correct insertion of the ORF fragments was checked using primers C2950 and A9050, insertion of terminator fragments was checked using primers B1222 and A2047.

Construction of *C. glabrata* strains

The trehalase deletion strains were constructed in the ATCC2001 *his3Δ trp1Δ leu2Δ* background (Fig S2) [59]. The wild type strain was transformed by electroporation with the deletion cassette (a nourseothricin cassette flanked by FRT sites and a 100 bp region flanking the targeted gene). The deletion cassettes were PCR amplified from the pYC44 plasmid using primers C6315 and C6316 (*ATH1*), C6317 and C6318 (*NTH1*) or C6319 and C6320 (*NTH2*). Cells were plated on YPD agar medium supplemented with 200 µg/mL nourseothricin. Transformants were checked for insertion of the deletion cassette by PCR using control primers C3177 and C3178 (*ATH1*), C3183 and C3184 (*NTH1*) or C3161 and B3162 (*NTH2*). Correct strains were subsequently transformed with plasmid pLS10 to induce expression of the flippase enzyme (300 µg/ml hygromycin selection). Removal of the nourseothricin cassettes of the transformants was checked by PCR using primers C3177 and C8497 (*ATH1*), C3183 and C3835 (*NTH1*) or C3161 and B2011 (*NTH2*). The pLS10 plasmid was lost by growth on nonselective YPD medium and checked by replating on YPD supplemented with 300 µg/mL hygromycin. The trehalase double and triple mutants were constructed similarly, by transformation of the nourseothricin deletion cassettes mentioned above in the single or double deletion strains, respectively, followed by removal of the selective marker by expression of pLS10. Endogenously tagged trehalase strains were constructed by electroporation of the wild type strain with *Eci*I digested plasmid pBM13 (*ATH1*-mCherry), pBM14 (*NTH1*-mCherry), or pBM15 (*NTH2*-mCherry). The resulting transformants were checked using primers C9381 (*ATH1*), C9385 (*NTH1*) or C9388 (*NTH2*), and A9050 (mCherry).

Growth phenotype

Growth of the strains was followed in time both in liquid and on solid YNB medium over time. Overnight cultures of the different strains were washed three times with sterile milli-Q water and subsequently diluted to an OD_{600} of 0.1. For growth assays in liquid medium, cells were grown in 50 mL YNB containing either 10 mM of glucose or 10 mM of trehalose as the carbon source. The cells were grown at 37°C with continuous shaking at 200 rpm for 72 hours during which the OD_{600} was monitored. For growth assays on solid medium, a tenfold dilution series of the cultures was spotted on YNB plates containing 5 mM of glucose or 5 mM of trehalose. To investigate the stress response, the washed overnight cultures were spotted on YNB plates supplemented with 100 mM glucose, containing either 6 mM H_2O_2, 1.5 M NaCl or 0.4 mg/mL CFW. The plates were incubated at 37°C for 72 hours during which growth was assessed.

Protoplast preparation

Overnight cultures of the different strains were made in YPD medium. Subsequently, the cultures were grown until mid-exponential phase in YPD (100 mM glucose) or YPT (100 mM trehalose). The cells were collected and washed twice, after which they were incubated for 15 to 45 minutes in the protoplasting solution (600 mM KCl, 800 mM Sorbitol, 41.7 mM K_2HPO_4, 8.3 mM KH_2PO_4, 4.8 units/mL Zymolase, 9.65 mM β-mercaptoethanol) until the OD_{600} dropped. The cells were collected and washed twice with cold protoplasting buffer (166.8 mM K_2HPO_4, 33.2 mM KH_2PO_4, 800 mM

Sorbitol), after which trehalase activity was determined as described below.

Determination of trehalase activity

Trehalase activity was determined as described in Pernambuco, 1996 [60]. In short, crude extracts were incubated with 50 µL of acid trehalose buffer (50 mM trehalose, 200 mM sodium citrate, 2 mM EDTA, pH 4.5). After 30 minutes of incubation at 30°C, the reaction was terminated by boiling for 5 minutes at 90°C. The glucose liberated was determined by the glucose oxidase–peroxidase method. Protein levels were determined by the Lowry method. Trehalase specific activity is expressed as nmol of glucose released per min and mg of protein.

Determination of extracellular compounds

Extracellular glucose and trehalose concentrations during growth were analyzed by the Shimadzu HPLC system using an Agilent 87 H column at 0.7 mL/min and a RID-20A detector (Shimadzu).

Fluorescence microscopy

We used a FluoView FV1000 confocal microscope (Olympus IX81) and its software for localization of Ath1. We visualized mCherry with a 559-nm laser and BA575-675 emission filter. A 60x UPlanSApo (numerical aperture [NA], 1.35) objective lens was used.

Acute hydrogen peroxide survival

Overnight cultures of the strains in SC 100 mM glucose were inoculated in 50 mL SC 100 mM. These cultures were allowed to grow for 48 hours at 37°C until reaching stationary phase. The cells were washed and diluted to OD_{600} 0,5 in YNB 100 mM containing different concentrations of H_2O_2 (0, 6, 10, 20, 50, and 100 mM). The cells were kept under stress conditions for 1 h at 37°C after which they were washed with water. The cells were diluted fivefold and spotted onto YPD plates which were placed at 37°C. Scans of survival were taken after 24 hours.

Ethical statement

Blood was obtained from healthy human volunteers with written informed consent according to the declaration of Helsinki. The blood donation protocol and use of blood for this study were approved by the Jena institutional ethics committee (Ethik-Kommission des Universitätsklinikums Jena, Permission No 2207–01/08).

Expression analysis

Short term analysis in murine cell line macrophages (RAW264.7)

5 X 10^6 RAW264.7 cells (murine macrophage cell line) were seeded in 14 mL DMEM + 10% FBS (PAA laboratories) into 100 mm diameter cell culture petri dishes (TPP, Techno Plastic Products) and incubated for two days with a medium exchange after one day. A *C. glabrata* ATCC2001 overnight culture was pelleted, taken up in DMEM + 100 µg/mL Ampicillin + 100 µg/mL Kanamycin and cell counts were determined. 108 *C. glabrata* yeast cells were added per petri dish and infected petri dishes were stored immediately for 30 min on ice for synchronization of phagocytosis. After 30 min, non-adhered yeast were washed away twice with PBS after which 14 mL DMEM + 100 µg/mL Ampicillin + 100 µg/mL Kanamycin was added and co-incubation at 37°C and 5% CO2 was started. Samples were taken after 0 (directly from ice), 10, 30, 60, 180, and 360 min: non-phagocytosed yeast were washed away twice with PBS and macrophages were lysed with AE buffer + 1% SDS. Centrifugation (2 min 12.000 g) was used for separation of the fungal cell pellet from macrophagal DNA and RNA. The fungal cell pellet was frozen in liquid nitrogen.

Long-term analysis in human monocyte-derived macrophages (hMDMs)

Method details are described in Fischer et al. (in preparation). Briefly, hMDM monolayers in RPMI + 10% HS were infected in cell culture flasks (Greiner) with *C. glabrata* ATCC2001 at an MOI of 20 and non-phagocytosed yeasts were washed away with PBS twice after 3 hours of co-incubation. Caspofungin was added after 6 hours of co-incubation and further held constant on a level of 5 µg/mL to hinder extramacrophagal yeast growth. Medium was in part exchanged daily. Samples were taken after 0,25, 1, 2, and 4 days widely similar as above (but with AE + 10% SDS).

RNA isolation, labeling and microarray analysis

Fungal RNA was isolated using a modified freeze-thaw protocol [61]. Optionally, β-mercaptoethanol was used at a final concentration of ≈ 5% in AE buffer for yeast resuspension. The QuickAmp Labeling Kit (Agilent) was used to generate Cy5-labeled cRNA (Cy5 CTP; GE Healthcare). Cy5-labeled samples were co-hybridized with a Cy3-labeled reference (RNA isolated from *C. glabrata* ATCC2001 grown to mid-log phase)

on 8-by-15 K format arrays (Agilent) and scanned either in Agilent´s High Resolution C Scanner with Scan Control (Agilent) or in a GenePix 4200AL with GenePix Pro 6.1 (Auto PMT, pixel size 5 μm). Microarray data were analyzed using GeneSpring 14.8 (Agilent).

Fungal killing by human macrophages

Differentiation of human monocytes into human monocyte-derived macrophages

Preparation of human monocyte-derived macrophages (hMDMs) was done as described previously [62]. Briefly, monocytes were selected from buffy coats by magnetic automated cell sorting of CD14 positive monocytes, seeded in 175 cm^2 cell culture flasks (Greiner) and differentiated over a time period of seven days. At day 7, hMDMs were detached, and 1.5×10^5 cells/well were seeded for infection in a 24-well plate in RPMI + 10% FBS (Gibco). The day after, the medium was exchanged to RPMI + 10% human serum (HS; from AB male donors; sterile-filtered, Bio&Sell) with an intermediate PBS washing step.

Macrophage infection with C. glabrata

For the preparation of yeast inocula, overnight cultures of *C. glabrata* strains were pelleted, washed twice with PBS and adjusted to 1.5×10^6 cells/mL. The macrophages were infected with 1.5×10^5 *C. glabrata* cells (multiplicity of infection (MOI) 1:1). Three hours after infection, the non-phagocyted cells were washed away and plated to check for phagocytosis efficiency and 1 mL of fresh RPMI + 10% HS was added per well. At this same time point, also the lysate was plated for the 3 h timepoint. At one and four days, the supernatant was removed without washing, and the hMDMs lysed and plated. From wells intended for four days co-incubation, 0.5 mL of supernatant was removed after one day and 1 mL fresh medium was added (total volume 1.5 mL). On each following day, 0.5 mL was replaced with fresh medium. All wells were constantly checked for appearance of yeast microcolonies by naked eye. Before macrophage lysis and yeast plating, each well was systematically checked for extracellular yeasts with an inverse microscope, and only wells that met the cutoff criteria were used for plating. CFU counts on YPD agar plates were determined manually.

In vivo mouse model of systemic infection

The virulence of the different strains was assessed in an *in vivo* mouse model of systemic infection in female BALB/c mice (8 weeks old). The mice were housed in groups of four in filter-top cages in a dedicated animal room where temperature, light, and humidity were regulated. The animals received a standard *ad libitum* diet and water. At day −3, all mice were immunosuppressed with 75 mg/kg dexamethasone (Fagron) through intraperitoneal injection. This concentration was chosen after a pilot study as the effect of the trehalase deletion on the infection can be assessed (more or less virulent). After this, the animals received the same amount of immunosuppression on day 0 and from then on, every seven days. On day 0, the mice were injected intravenously via the lateral tail vein with 5×10^7 *C. glabrata* cells in 200 μL PBS. After this, the infected mice were monitored daily and when they reached humane endpoints, they were euthanized by cervical dislocation under anesthesia. The survival assay was terminated at day 18 after infection.

In vivo mouse model of gastrointestinal colonization

The gut colonization capacity of the different strains was determined in an *in vivo* mouse model of gastrointestinal colonization in female c57BL6/J mice (8 weeks old). The mice were housed in groups of four in filter-top cages in a dedicated animal room where temperature, light and humidity were regulated. The animals received a standard *ad libitum* diet and water. At day 0, the mice received 10^8 *C. glabrata* cells via oral gavage. From this day on, stool samples from each mouse were collected during 21 days at the timepoints indicated and plated on CHROMagar™ for CFU counting. The GI colonization is expressed as log(CFU/gram of stool). The gastrointestinal colonization model was terminated at day 21 after gavage. The animals were sacrificed by cervical dislocation under anesthesia and duodenum, ileum, cecum, proximal colon, distal colon, tongue and kidneys were plated for CFU counting on CHROMagar™.

Results

C. glabrata trehalase enzymes are phylogenetically closely related to *S. cerevisiae* trehalase enzymes

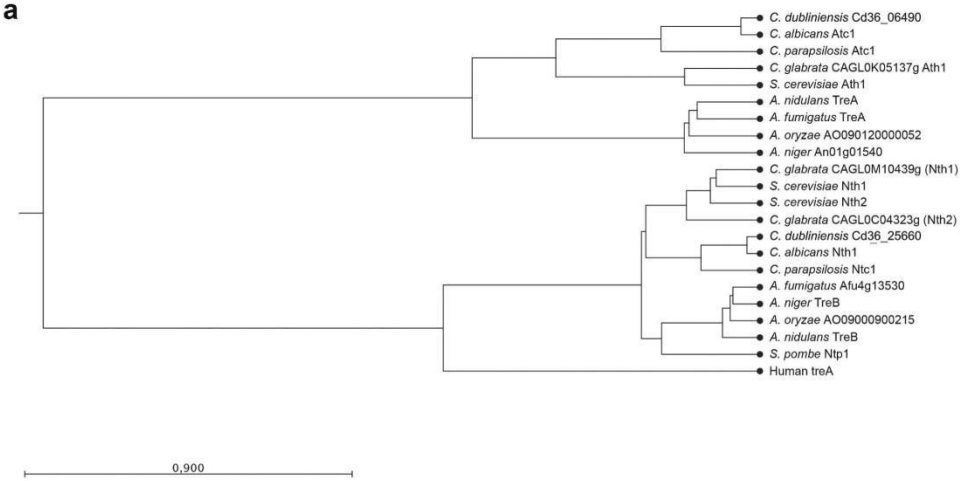
Despite what its name suggests, *C. glabrata* is more related to species within the *Saccharomycetaceae* clade than to species within the *Candida* clade [63,64]. Therefore, we used the *S. cerevisiae* trehalase protein sequences in a BLASTP search to identify the *C. glabrata* trehalase enzymes. In this manuscript, genes/proteins of other species than *C. glabrata* are indicated with a prefix. For the neutral trehalases *Sc*Nth1 (YDR001C) and *Sc*Nth2

(YBR001C), the *C. glabrata* Nth1 (CAGL0M10439g) and Nth2 (CAGL0C04323g) were identified as closest orthologs respectively. These proteins are highly conserved to their *S. cerevisiae* orthologs, as reflected by the high percentage of amino acid identity (80% for Nth1 and 68% for Nth2) (Figure 1b). As is the case for *S. cerevisiae*, Nth1 and Nth2 are highly similar with one another (71% amino acid identity), indicating that these enzymes are most probably the result of the whole genome duplication event [65]. *NTH1* encodes for a polypeptide of 769 amino acids with a molecular mass of 87.4 kDa, while *NTH2* encodes for a polypeptide of 750 amino acids with a molecular mass of 86.5 kDa. Both Nth1 and Nth2 amino acid sequences contain neither a transmembrane domain nor a signal sequence [66,67]. Alignment of Nth1 with *Ca*Nth1 (CR_00560W_A) shows 58% amino acid identity, reflecting the more distant phylogenetic relationship between *C. glabrata* and *C. albicans*.

For the acid trehalase *Sc*Ath1 (YPR026W), Ath1 (CAGL0K05137g) was found as an ortholog in *C. glabrata*, sharing 67% amino acid identity. *ATH1* encodes for a polypeptide of 1212 amino acids and 136.5 kDa. In contrast, alignment of Ath1 with *Ca*Atc1 (C1_06940C_A) showed only 41% amino acid identity (Figure 1b). In other organisms, Ath1 was often found on the extracellular side of the cells [37,39,40,51,68,69]. Hence, the PROTTER and the SignalP4.1 algorithms were used to predict a possible transmembrane domain or a signal sequence [66,67]. No signal peptide was found, but Ath1 is predicted to contain one transmembrane (TM) domain at the N-terminus (between positions 83 and 103). The orthologous N-terminus and TM domain of *Sc*Ath1 confers the extracellular localization, suggesting identical extracellular localization for Ath1 in *C. glabrata* [37].

To represent the evolutionary relationship, we constructed a phylogenetic tree of the different fungal trehalase enzymes and also included the human trehalase enzyme (Figure 1a). Two main clusters can be distinguished: the neutral trehalases and the acid

a



b

C. glabrata trehalase	*S. cerevisiae* trehalase	Percentage identity
Nth1 (CAGL0M10439g)	Nth1 (YDR001C)	80%
Nth2 (CAGL0C04323g)	Nth2 (YBR001C)	68%
Ath1 (CAGL0K05137g)	Ath1 (YPR026W)	67%

Figure 1. Phylogenetic tree of fungal trehalases. Two main clusters can be distinguished: the neutral trehalases and the acid trehalases. The human trehalase enzyme was included and is present in the cluster of neutral trehalase enzymes. Protein sequences were aligned and the phylogenetic tree was constructed using the unweighted pair method with arithmetic mean (UPGMA) and the Kimura Protein as a protein distance measure (a). Overview of amino acid identity between the *C. glabrata* and *S. cerevisiae* trehalases (b).

trehalases. The *C. glabrata* neutral trehalases Nth1 and Nth2 are closely related to *S. cerevisiae Sc*Nth1 and *Sc*Nth2 respectively. The acid trehalase Ath1 is most related to *Sc*Ath1. Again, this is not unexpected as these species share a close evolutionary relationship [63,64].

Ath1 is required for growth on trehalose as a carbon source

In order to study the *C. glabrata* trehalase enzymes, we generated the single-, double- and triple trehalase deletion strains as described in Materials and Methods. As a first step of the *in vitro* characterization of the constructed mutants, we tested the growth of the strains in minimal medium supplemented with glucose or trehalose, two carbon sources that *C. glabrata* is able to ferment [70]. All strains grew to the same extent on glucose in both solid and liquid medium (Figure 2(a,b)). When trehalose was used as external carbon source, the growth of all strains lacking *ATH1* had a growth defect in both liquid and solid medium (Figure 2(a,c)). These results indicate that Ath1 is responsible for the hydrolysis of exogenous trehalose, which led to two hypotheses: either *C. glabrata* transports extracellular trehalose to the inside of the cell by a trehalose transporter and Ath1 hydrolyzes the disaccharide intracellularly, or Ath1 is present at the extracellular side of the cells and hydrolyzes trehalose in the surrounding medium after which glucose is taken up by the glucose transporters. In *S. cerevisiae*, extracellular trehalose is transported by the high-affinity sugar transporter *Sc*Agt1 [71]. Therefore, a BLASTP search was performed using *Sc*Agt1, however no orthologs could be identified in *C. glabrata*. Hence, we measured the concentration of trehalose in the medium over time during the growth on trehalose. We postulated that if Ath1 does indeed hydrolyze trehalose intracellularly after uptake by transporters, a strain deleted for *ATH1* would still show reducing extracellular trehalose levels over time. Yet, for the strains with a deletion of *ATH1*, the trehalose in the medium remained constant (Figure 2d). In contrast, the strains with wild type *ATH1* could still hydrolyze the extracellular trehalose, which is reflected by a drop of trehalose in the medium (Figure 2d). Additionally, coinciding with this decrease in trehalose, a small increase of glucose in the medium was observed, confirming extracellular hydrolysis of trehalose in two glucose molecules (Figure 2e). Taken together, these results suggest that Ath1 hydrolyses extracellular trehalose and thus indicate that Ath1 is present at the cell surface or is secreted. Therefore, we determined its localization by tagging Ath1 endogenously with a fluorescent protein, mCherry. When *C. glabrata* was grown in glucose-containing medium to the exponential phase, no fluorescence was detected. However, when trehalose was used as a carbon source, a signal on the border and in the vacuole of the cells was observed (Figure 3a and Fig S3). Next, we investigated whether Ath1 is anchored in the plasma membrane as Ath1 contains one predicted TM domain. Therefore, we generated protoplasts of cells grown in glucose or trehalose containing medium and measured the trehalase activity. The basal activity of the cells grown on glucose is very low and is not affected by protoplast formation. When grown on trehalose, the high trehalase activity observed in untreated cells, dropped dramatically in protoplasts (Figure 3b). In addition, the fluorescent signal observed on the outside of untreated cells, was no longer visible in protoplasts (Figure 3a). These results indicate that Ath1 is not anchored to the cell membrane but is rather present in the periplasmic space or in the cell wall.

The triple deletion strain is unable to stably colonize the gut

As humans cannot produce trehalose, its presence in the gut originates from ingested food or from micro-organisms as a result of production or release after microbial death. As Ath1 is required for growth on extracellular trehalose, we hypothesized that this enzyme could give *C. glabrata* cells a competitive advantage over other micro-organisms. To test this hypothesis, we verified whether the wild type and different deletion strains were able to colonize the gut over a longer period of time in a mouse model. To achieve *Candida* colonization in the gut, mice are frequently treated with antibiotics [72,73]. As we want to assess the competition with these other micro-organisms, a pilot experiment was performed in non-treated mice and mice which received ampicillin in their drinking water (1 mg/ml) infecting them via oral gavage with wild type *C. glabrata* (Fig S4). Despite the higher colonization in mice receiving ampicillin, we also obtained a stable colonization in mice where microbial perturbation was not induced (Fig S4). Because of the higher relevance of the latter model, we continued the experiments in untreated mice and tested the trehalase single deletion strains and the triple deletion strain (Figure 4). Similar to the pilot experiment, a stable colonization was obtained for the wild type strain (Figure 4a). The main habitat of *Candida* in the GI tract was in the cecum, which had a log (CFU/g) of around 3 (Figure 4b). The single deletion strains showed a similar colonization as the wild type strain (Figure 4a). The triple deletion strain was not able to

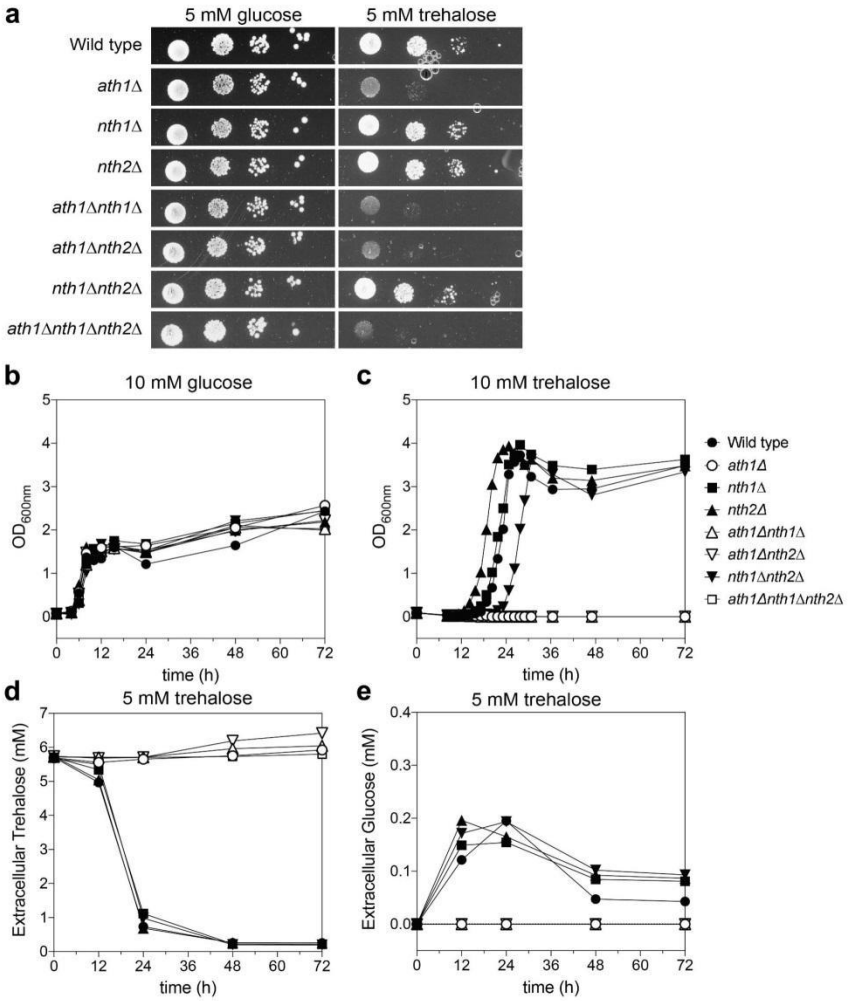


Figure 2. Growth of the different trehalase deletion strains. Cells were grown on YNB solid (a) and in liquid (b-e) medium containing glucose (a-b) or trehalose (A, C-E) as a fermentable carbon source. During growth in YNB trehalose, we also measured the trehalose and glucose concentrations in the medium over time (D-E, respectively). The experiments were performed at least twice and average results of three independent transformants are shown.

establish a stable colonization of the GI-tract, as over 66% of the mice cleared the *C. glabrata* administered at day 21. This trend is clearly reflected in the right panel of Figure 4a. Logically, the average colonization of the cecum of mice which were orally infected with the triple deletion strain was significantly lower, as over 66% of the mice showed no colonies (Figure 4b).

Nth1 is involved in regulation of *in vitro* oxidative stress resistance and survival in human macrophages.

Inside the human body, fungal pathogens are continuously exposed to different types of environmental stresses [74]. As trehalose is a disaccharide important for stress resistance, we assessed the growth phenotype of the trehalase

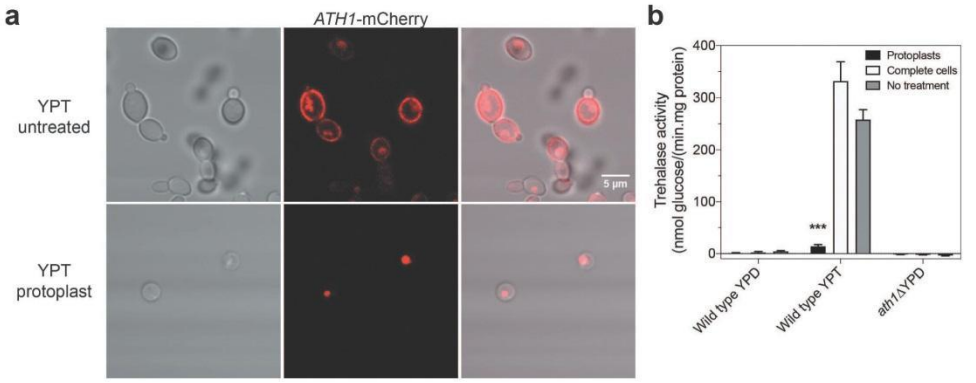


Figure 3. Ath1 is present in the periplasm and is responsible for high trehalase activity when cells are grown in presence of trehalose. Imaging of Ath1-mCherry cells taken with a Fluoview 1000 confocal microscope (a). Cells were grown in presence of trehalose to exponential phase after which protoplasts were made. The upper panel of A shows complete cells, the lower panel shows protoplasts of Ath1-mCherry. The trehalase activity was measured in complete cells, protoplasts and cells with no treatment after growth on YPD or YPT (b). Average trehalase activity of at least two experiments is shown with the standard error of the mean (SEM). Statistical Kruskal-Wallis test was used with Dunn's correction for comparing treated and untreated conditions for each strain (*, P ≤ 0,05; **, P ≤ 0,01; *** P ≤ 0,001 and ****, P ≤ 0,0001).

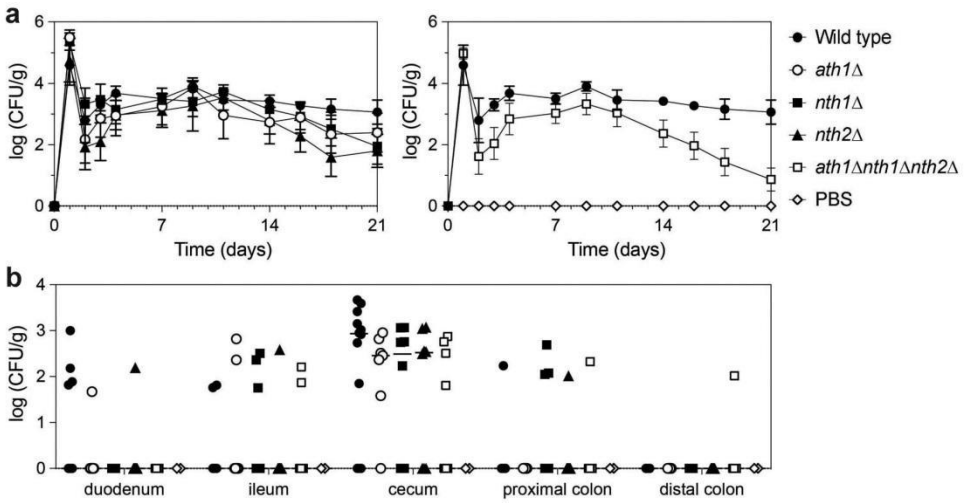


Figure 4. Colonization in the GI tract is not maintained in the triple trehalase deletion strain. At day 0, mice are orally infected with 10^8 *C. glabrata* cells (gavage). Wild type (●), *ath1*Δ (○), *nth1*Δ (■), *nth2*Δ (▲) and *ath1*Δ*nth1*Δ*nth2*Δ (Δ) or PBS as a control (□) were tested. At the indicated days a stool sample was collected from each mouse and plated for CFU counting, during 21 days (a). Upon termination of the experiment, mice were sacrificed and different tissues were collected to plate for CFU counting (b). All data are expressed in Log10 values.

deletion strains upon stress treatment. Therefore, cells were grown in the presence of different relevant stresses: oxidative stress (H_2O_2), salt stress (NaCl) and cell wall stress (Calcofluor White, CFW). Deletion of *ATH1* and/or *NTH2* did not affect the growth in the presence of these stressors, while strains lacking *NTH1* were more sensitive toward oxidative stress (Figure 5, Fig S5 and Fig S6). This phenotype is observed in all strains lacking *NTH1* with no additive effect of the other trehalases (Fig S6). Furthermore, all *nth1Δ* strains showed a decreased survival after acute exposure to different concentrations of hydrogen peroxide (Fig S7). The disruption of *NTH1* did not affect growth in the presence of salt nor cell wall stress (Fig S5).

One of *C. glabrata*'s virulence traits is its ability to survive and replicate inside human macrophages [75–78]. This survival demands for a high stress resistance as the yeast cells encounter a change in pH, nutrient limitation and oxidative stress within the macrophages [79,80]. As a difference in oxidative stress resistance was observed for the *nth1Δ* strains, their survival in macrophages was investigated. In addition, the lab of prof. Hube at the Hans Knöll Institute, Jena, Germany investigated the transcriptional response of *C. glabrata* cells to incubation with a macrophage like-murine RAW cell line. The transcriptome showed that in comparison to growth in complex medium, the expression level of the acid trehalase gene *ATH1* was overall very high, slightly dropped during the first hour and went up again (Fig S8A). Furthermore, the expression of *NTH1* and *NTH2* increased within the first hour of incubation, with the transcript level especially of *NTH2* remaining very high at all later timepoints inside the macrophage-like cells (Fig S8A). We therefore also tested if the deletion of the trehalase enzymes could influence the survival in human macrophages, especially at later timepoints, when trehalose utilization may play an important role. To this end, we infected human monocyte-derived macrophages with the different *C. glabrata* strains at a multiplicity of infection (MOI) of 1:1 for a long-term co-incubation. At different timepoints (3 hours, 1 day and 4 days), the macrophages were lysed and the *C. glabrata* cells were used for transcript determination by microarrays and plated for CFU counting to determine survival (Fig S8B and 6A-D). To ensure the difference in survival was due to killing by macrophages and not to altered uptake or escape and extracellular growth of the *Candida* cells, we measured the uptake rate and ensured that our samples did not contain extracellular *C. glabrata*. The uptake by the immune cells was nearly identical for all strains, with a somewhat increased uptake of *nth2Δ* (Figure 6a). After 3 hours, more *nth1Δ* than wild type yeasts were re-isolated (not statistically significant), while the *nth2Δ* and the triple deletion mutant were found to be slightly reduced in surviving numbers. These small differences were gone after

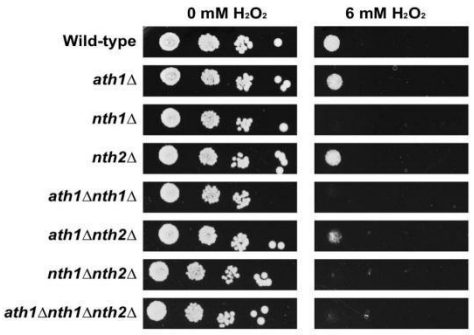


Figure 5. Oxidative stress resistance of different trehalase mutant strains. The different strains were grown overnight in SC 100 mM glucose. After 3 washing steps, cells were spotted on YNB plus 100 mM glucose agar plates containing 6 mM of H_2O_2 and grown at 37°C for 72 hours.

one day of incubation, with the strains adapting to long-term survival (Figure 6c). After 4 days of incubation, all single deletion strains and the triple deletion strain showed a significantly decreased survival (down to 44% for *ath1Δ*, 65% for *nth1Δ*, 35% for *nth2Δ* and 64% for *aht1Δ nth1Δ nth2Δ*) compared to the wild type strain (Figure 6d). The requirement for these genes in human macrophages was reflected by the continuously increased transcript levels as determined in parallel by microarray experiments (Fig S8B).

Trehalase plays only a minor role in the virulence of *C. glabrata* in a mouse systemic infection model

In order to investigate whether the trehalase enzymes are involved in *C. glabrata* virulence in a systemic infection, mice were infected intravenously with the different deletion strains. Experimental infection of immunocompetent mice with *C. glabrata* generally does not cause mortality [81]. We confirmed this in a pilot experiment during which we determined the optimal concentration of the immunosuppressant dexamethasone (Fig S9). 75 mg/kg dexamethasone was chosen to be given to the mice at day −3, 0, 7 and 14. For each deletion strain, at least 8 mice were included in the experiment (Figure 7). The mice infected with the *nth1Δ* strain appeared ill more rapidly and died earlier compared to the wild type (p-value of 0.0184 in log-rank test). The mice infected with the triple deletion strain showed no significant difference in survival compared to mice infected with the wild type strain (p-value 0.0873 in log-rank test). Based on the twice per day monitoring, it was clear that mice infected with the triple deletion mutant did not show

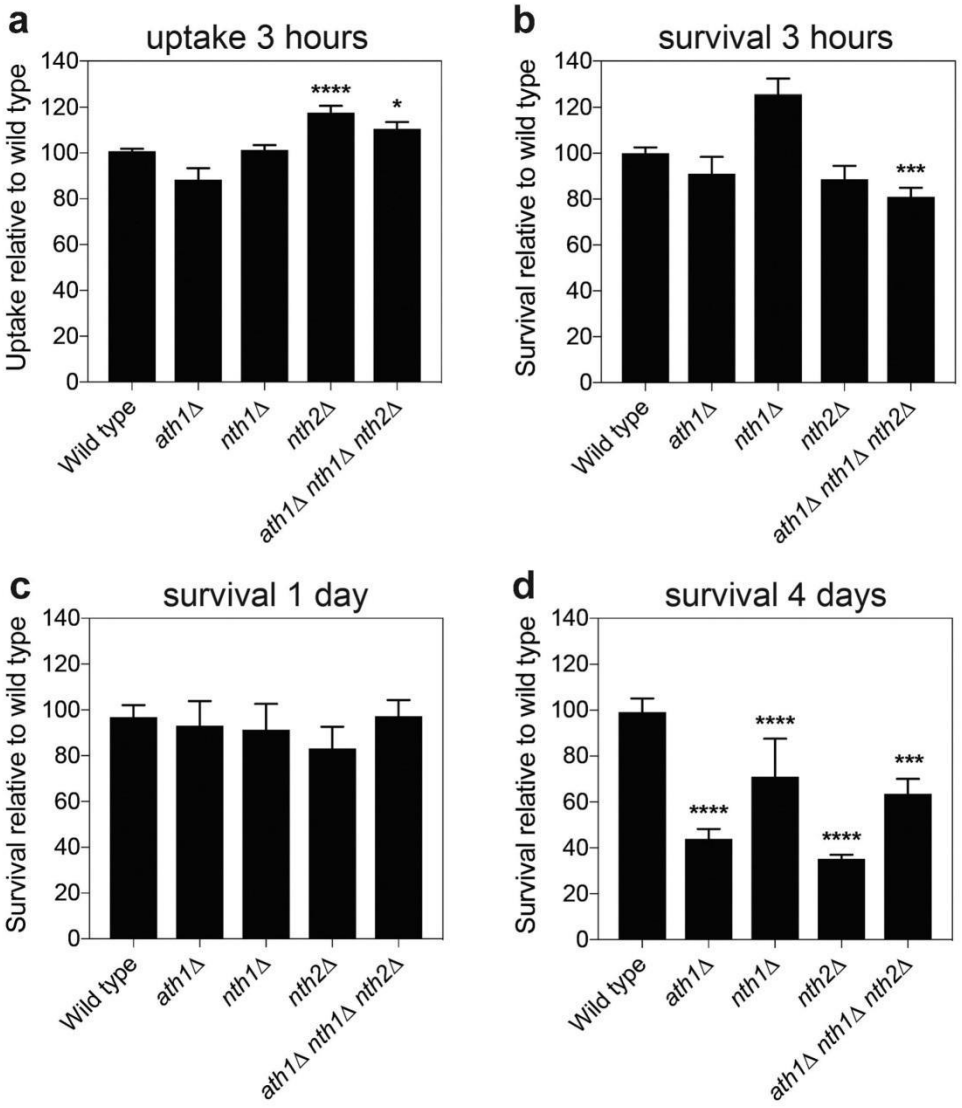


Figure 6. *Candida glabrata* trehalase survival inside macrophages. Differentiated human macrophages were infected with the indicated *C. glabrata* strain at multiplicity of infection (MOI) 1:1, after which the cells were plated for CFU counting at the indicated days (b-d). Additionally, at 3 hours post infection, also the uptake of each strain was counted (a). Average survival relative to the wild type strain is shown with SEM of minimal three experiments, statistical Kruskal-Wallis test was performed with Dunn's correction (*, $P \leq 0,05$; **, $P \leq 0,01$; *** $P \leq 0,001$ and ****, $P \leq 0,0001$), comparing the survival of all strains to wild type.

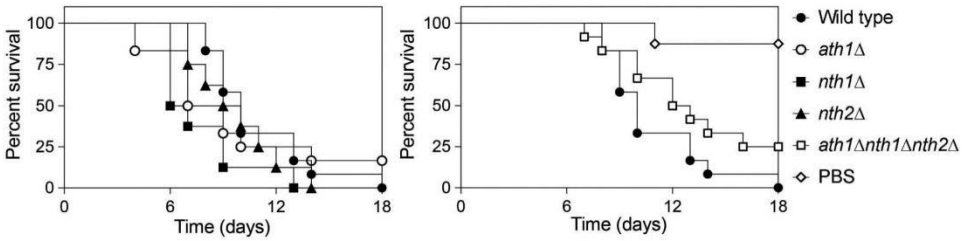


Figure 7. Mice survival after systemic infection. Mice were immunosuppressed with 75 mg/kg dexamethasone and received 5 . 10^7 *C. glabrata* cells via injection in the lateral tail vain. Wild type (●), *ath1*Δ (○), *nth1*Δ (■), *nth2*Δ (▲) and *ath1*Δ*nth1*Δ*nth2*Δ (Δ) or PBS as a control (□) were tested. The mice were monitored two times per day for 18 days. When they reached humane endpoints, they were sacrificed. Statistical analysis by the use of a log-rank test was performed comparing the deletion strains to the wild type (*, P ≤ 0,05; **, P ≤ 0,01; *** P ≤ 0,001 and ****, P ≤ 0,0001).

disease phenotypes such as ruffled hair, similar to the PBS control mice and different from mice infected with the wild type strain. It should be noted that two mice of the PBS control group died, which is most probably due to the immunosuppression of the mice and the duration of the experiment. The other mice of the PBS control group showed a healthy appearance throughout the experiment.

Discussion

This work focused on the characterization of the different *C. glabrata* trehalase enzymes and their role in virulence. To our knowledge, there are no prior publications in which a mutant lacking all three trehalase enzymes in a pathogenic yeast strain is characterized. Comparison with the *S. cerevisiae* trehalase enzymes resulted in the identification of three trehalase enzymes in *C. glabrata*: Ath1, Nth1 and Nth2. When comparing the *C. glabrata* trehalase enzymes to those present in other pathogenic and nonpathogenic fungi two clusters can be distinguished: a cluster of neutral trehalase enzymes and a cluster of acid trehalase enzymes. Both types of these proteins have a different structure: the neutral trehalases contain one large domain for trehalase activity containing the active site and binding site for the substrate, next to a small calcium binding domain. The acid trehalase contains one predicted transmembrane domain and two domains for trehalase activity [82].

Single and multiple deletions of the encoding genes were constructed and functionally characterized. We showed that Ath1 is important for the rapid hydrolysis of extracellular trehalose, a phenotype on which the rapid identification of *C. glabrata* in the hospital is based [42–48]. In different organisms, the acid trehalase enzyme is present on the outside of the cells in order to hydrolyze exogenous trehalose [37,39,40,51,68,69]. Our microscopy data, where Ath1 was tagged to a fluorophore, showed that Ath1 is present both on the outside of the cells as well as in the vacuole. Furthermore, high acid trehalase activity was measured in complete cells, which was lost upon preparation of protoplasts. At the same time, the fluorescent signal of the labeled Ath1 at the outside of the cells is lost. This shows that Ath1 is localized in the periplasmic space, similar to the situation in *S. cerevisiae* [37]. We hypothesize that this localization helps the cells to quickly hydrolyze extracellular trehalose to glucose, which is then taken up by the glucose transporters in the plasma membrane [83]. Zilli et al. showed that Ath1 is also secreted to the outside of the cells and is released into the medium [41]. As the activity of Ath1 is very high, we hypothesized that *C. glabrata* cells would have a competitive advantage over other microbes in those conditions where there is trehalose present, such as in the gut. In humans, *Candida* is present in the gastrointestinal tract as part of the normal microbiota [84]; hence, we hypothesized that Ath1 might play a relevant role in the colonization of *C. glabrata* at this body site. This appeared not to be the case, as in our experiments, the *ath1Δ* strain showed a wild type phenotype in the mouse model of GI colonization. Interestingly, additional deletion of *NTH1* and *NTH2* resulted in a clear drop in GI colonization. This indicates that trehalase activity is indeed important to achieve persistent colonization, however, any of the enzymes could contribute to this.

In *C. albicans* and in *C. parapsilosis*, the acid trehalase Atc1 is a stress-responsive protein and is

important for *in vitro* stress resistance. Moreover, mice infected intravenously with *C. albicans* or *C. parapsilosis* cells lacking their acid trehalase enzyme show an increased survival, suggesting a role for *ATC1* in virulence [49,51,85]. None of these phenotypes are observed for the acid trehalase of *C. glabrata*. However, after 4 days of infection in macrophages, Ath1 seemed to play a role in the survival of *C. glabrata* cells. We hypothesize that through binding to trehalose as a stress protectant, many proteins are cycled to the vacuole for autophagy [77]. As such, it is likely that Ath1 is present in the vacuole which allows trehalose hydrolysis in the vacuole, as is the case in *S. cerevisiae* [37], providing energy to the cells. These data confirm that the function of Ath1 is different from its orthologous enzymes in other *Candida* species, possibly explaining why the specific trehalose test is used to diagnose *C. glabrata*.

Similar to *S. cerevisiae, C. glabrata* Nth1 is important for stress tolerance, specifically toward oxidative stress, which is encountered inside immune cells. Deletion of *NTH1*, as well as the other trehalases, indeed results in a lower survival upon engulfment by macrophages. We hypothesize that the reason for this is the lower tolerance toward oxidative stress for this deletion strain. The small enhanced virulence of the *nth1Δ* strain in the systemic mouse model was unexpected. However, other examples are known, where a mutant strain shows opposite phenotypes in different virulence experiments [86,87]. Important to mention is the fact that the mice in the experiment are immunocompromised, leaving out a role for the immune system, in which the trehalase deletion strains would survive less well. The role of Nth2 remains unclear. Under *in vitro* conditions, the *NTH2* gene is rapidly upregulated inside macrophages and its deletion results in a lower survival under these conditions. Its higher expression in macrophages resembles the higher *ScNTH2* expression during the onset of stationary phase growth [88]. This suggests that the cells encounter conditions in the macrophages similar to the ones they encounter when reaching stationary phase. The decreased survival of the mutant strain after 4 days remains to be investigated.

The *ath1Δ nth1Δ nth2Δ* strain did not show detectable trehalase activity in any growth phases, indicating that these three genes are all the trehalase enzymes present in *C. glabrata*. In general, the triple deletion strain showed results consistent with the single deletion strains. The triple deletion strain showed a decreased survival on hydrogen peroxide due to the absence of *NTH1*. This strain is also impaired in survival in human macrophages after 4 days of inoculation. Additionally, the triple deletion strain was unable to use trehalose as a carbon source, as is also the case for the *ATH1* deletion strains.

Based on our animal experiments, we can conclude that none of the single enzymes alone constitutes an interesting antifungal drug target, however a combined inactivation of these enzymes is of interest as the triple deletion showed a decreased survival in human macrophages and was fully cleared from the murine GI tract. Additionally, the mice infected intravenously with the triple deletion strain appeared more fit (based on our monitoring of their behavior) compared to the wild type control. As they share trehalose as a substrate, variants of trehalose may cause inhibition of all three enzymes. As far as the single targets are concerned, inactivation of Ath1 may already be interesting, as this enzyme is required for cells to grow on trehalose and its deletion results in a decrease in survival inside macrophages.

C. glabrata infections are frequent in immunocompromised patients, where the endogenous *C. glabrata* can disseminate from the GI tract to cause invasive infection [89]. These results seem promising and therefore, we propose that a drug targeting all three trehalases could have a positive effect on the outcome of a *C. glabrata* infection. As all enzymes have the same substrate, the search for a competitive inhibitor seems the most straight-forward. Structural analogues of trehalose can act as a competitive inhibitor of trehalase [90]. Validamycin A, a well-known trehalase inhibitor, is already used in food crops to prevent fungal infections [91]. This compound showed to have weak antifungal activity against *C. albicans* whereas a strong effect was observed against *A. flavus* [92,93]. Based on this work, we propose to test different structural analogues of trehalose for their antifungal activity against *C. glabrata*.

Even though humans cannot synthesize trehalose, their genome encodes for one trehalase. The human trehalase enzyme can be found in the intestines and kidneys but also in the urinary tract, where it is used as a marker for renal tubular damage [94,95].The human enzyme clusters together with the fungal neutral trehalases (Figure 1). It shows only 27% amino acid identity to both Nth1 and Nth2 of *C. glabrata*, but the amino acids involved in the substrate binding and the active site are conserved (supplementary data). However, a deficiency of the human trehalase is rare and causes diarrhea due to an intolerance to trehalose enriched food, such as mushrooms [96]. This should be taken into account when looking for competitive inhibitors of the *C. glabrata* trehalase enzymes.

Acknowledgments

M.V.E. and B.T. prepared the manuscript and performed the experiments. S.W. performed the mouse macrophage

experiments in the lab of K.K. We thank the labs of K.K. and S.B. for the collaborations on the macrophage experiments. We thank Alejandro De Las Peñas and Irene Castaño for kindly sending us plasmids. We acknowledge Nico Vangoethem for assistance in preparation of the figures. All authors contributed to discussion of the results.

This work was supported by grants from the Fund for Scientific Research Flanders under Grant G0F8519N and WO.009.16N and by the Research Council of the KU Leuven under Grant C14/17/063. S.W., G.V and O.R were supported by personal research grants from the FWO (Fund for Scientific Research Flanders) under Grants 1S91118N; 1S43720N and 1S55817N respectively.

Disclosure statement

The authors report no conflict of interest.

Funding

This work was supported by the Research Fund of KU Leuven (BE) [C14/17/063]; Fund for Scientific Research Flanders (FWO) [1S91118N]; Fund for Scientific Research Flanders (FWO) [WO.009.16N]; Fund for Scientific Research Flanders (FWO) [1S55817N]; Fund for Scientific Research Flanders (FWO) [G0F8519N]; Fund for Scientific Research Flanders (FWO) [1S43720N].

Ethical statement

Animals were maintained in accordance with the KU Leuven animal care guidelines and all animal experiments were approved by the Ethical Committee for Animal Experimentation of the KU Leuven (project numbers P061/2019 and P010/2020).

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6.5 Manuscript V

Acetate assimilation is an integral part of *Candida glabrata* persistence within the human host's macrophages

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Summary:

Candida glabrata is an opportunistic pathogen able to persist and replicate within the phagosome of macrophages, where it can use alternative carbon sources, such as carboxylic and fatty acids, to survive and replicate. Acetate transporters and channels might be important for *Candida glabrata* pathogenicity as it has been shown that the metabolism of acetate modulates immune recognition of this yeast. This study aims to analyze the role of *C. glabrata* acetate transporters and channels during interaction with host macrophages. Deletion mutants were subjected to human monocyte-derived macrophages in a long-term experiment where they showed an increased survival within the phagocytes. This work provides information on the metabolic processes that support *C. glabrata* survival and persistence within macrophages, and the potential antivirulence function of acetate metabolism during long-term phagosomal residence of this fungus.

Status: In preparation.

The candidate is:
☐ First author ☐ Second author ☐ Corresponding author ☒ Coauthor

Estimated authors' contributions:

Author	Conception	Data analysis	Experimental	Writing	Provision of the material
RA	75%	50%	50%	70%	
SSL		10%	20%	10%	
CLD			10%		
BT			5%		
MVE			5%		
SB		5%	10%		20%
MC		15%			20%
PVD		10%			30%
SP	25%	10%		20%	30%

Acetate assimilation is an integral part of *Candida glabrata* persistence within the human host's macrophages

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Abstract

Candida glabrata is considered to be a commensal yeast that has evolved distinct strategies to survive and proliferate within the human host, thereby becoming an important opportunistic pathogen. This fungus is able to persist and replicate within the phagosome of macrophages, where alternative carbon sources, such carboxylic and fatty acids are abundant and may be used by this pathogen to survive and replicate. Previously it has been shown that the metabolism of acetate influences biofilm formation and antifungal drug resistance, and it also modulates immune recognition of this fungus. Acetate transporters and channels might therefore be important for *Candida glabrata* pathogenicity. This work investigates the role of *C. glabrata* acetate transporters and channels during interaction with host macrophages. With this aim, deletion mutants were subjected to human monocyte-derived macrophages in a long-term experiment. We found that deletion mutants of acetate transporters showed an increased survival during long-term residence in phagocytes, which sheds light on the metabolic processes that supports *C. glabrata* survival and persistence within macrophages, unveiling a potential antivirulence function of acetate metabolism during long-term phagocytosis of this fungus.

Key words: Fungal pathogenesis, phagocytosis, acetate tranporters, antivirulence factors.

Introduction

Pathogenic fungi like *Candida glabrata* have evolved distinct strategies to survive and proliferate within the human host, including sophisticated mechanisms to rapidly adapt to a diverse range of environmental stresses (Seider, Heyken *et al*. 2010, Seider, Gerwien *et al*. 2014, Kasper, Seider *et al*. 2015, Miramon and Lorenz 2017). During gastrointestinal and vaginal colonization, glucose is scarce and alternative, nonfermentable carbon sources, mostly acetate and lactate (Yamaguchi, Sonoyama *et al*. 2005, Amabebe and Anumba 2018) may support growth, proliferation and survival of *Candida*. This also presumably happens within the phagosome, as many studies show an upregulation of genes involved in carboxylic acids metabolism during phagocytosis (Lorenz, Bender *et al*. 2004, Danhof, Vylkova *et al*. 2016). Adaptation of pathogenic fungi to these environments requires dramatic transcriptional and metabolic changes, including a switch to a respiratory metabolism and the overexpression of carboxylate transporters to assimilate these nonfermentable carbon sources. Some components of these metabolic pathways are required for full virulence in animal models of candidiasis (Lorenz and Fink 2001, Barelle, Priest *et al*. 2006, Ramirez and Lorenz 2007, Chew, Ho *et al*. 2019), highlighting the importance of nutrient assimilation for the success of these yeasts as human pathogens.

C. glabrata is able to persist and replicate within the phagosome of macrophages, where alternative carbon sources, such carboxylic and fatty acids are abundant and may be used by this pathogen to survive and replicate (Lorenz, Bender *et al*. 2004, Seider, Heyken *et al*. 2010, Seider, Gerwien *et al*. 2014, Kasper, Seider *et al*. 2015). Previously it has been shown that the metabolism of acetate influences biofilm formation and antifungal drug resistance, and it also modulates immune recognition of this fungus (Mota, Alves *et al*. 2015). The putative acetate transporter gene, *ADY2a* (from here on referred to as Acetate Uptake Transporter: *ATO*) is upregulated in *C. glabrata* during interaction with macrophages (Kaur, Ma *et al*. 2007) and neutrophils (Fukuda, Tsai *et al*. 2013). The same is the case for the homologue of the *S. cerevisiae* acetate channel gene, *FPS1*, during interaction with macrophages (Seider, Heyken *et al*. 2010). Therefore, acetate transporters and channels might be important for *C. glabrata* pathogenicity via their functions in immune cell interactions. However, it was also observed that acetate-grown *C. glabrata* cells are better phagocytosed and killed than cells grown in glucose, showing that the presence of acetic acid is not necessarily advantageous for fungal cells to deal with macrophage defences (Mota, Alves *et al*. 2015).

This work aims to provide insight into the role of *C. glabrata* acetate transporters and channels during interaction with human macrophages. First, we show that each *C. glabrata* Ato protein can complement the loss of ScAto function in baker's yeast. In parallel, deletion of these genes significantly diminished the uptake of acetate by *C. glabrata*. Wild type and mutants were then subjected to human monocyte-derived macrophages in a long-term experiment to study the physiological role of these transporters and channels. We found that, although some of the genes were upregulated in the wild type after long periods of phagocytosis, their deletion mutants showed an even increased survival during long-term residence in phagocytes. This sheds light on the metabolic processes that support *C. glabrata* survival and persistence within macrophages, making these transporters potential antivirulence genes.

Results

Construction of *C. glabrata* mutants of putative acetate transporters and channels

C. glabrata ATO1 (systematic identifier CAGL0M03465g), *ATO2* (CAGL0L07766g), and *ATO3* (CAGL0A03212g) were annotated as putative acetate plasma membrane transporter genes based on the sequence similarities of their encoded proteins (protein BLAST identities of 74.30%, 73.66%, and 31.07%, respectively) to the *S. cerevisiae* acetate transporter Ato1 (YCR010C) (Paiva, Devaux *et al*. 2004, Ribas, Soares-Silva *et al*. 2019). *C. glabrata FPS1* (CAGL0C03267g) and *FPS2* (CAGL0E03894g) were annotated as putative acetate channel genes based on the sequence similarities of their encoded proteins (protein BLAST identities of 53.79% and 46.94%, respectively) to the *S. cerevisiae* aqua(glycerol)protein Fps1 (YLL043W), involved in acetic acid uptake (Mollapour and Piper 2007). In *C. glabrata*, these genes were previously observed to be upregulated in the presence of acetic acid at pH 5.0 (Mota, Alves *et al*. 2015), supporting their role as potential acetate transporters or channels.

To determine the physiological function of these proteins, each gene was successively deleted in the *C. glabrata* Δ*HTL* strain by using the *SAT1* flipper method (Figure 1A). Two rounds of integration/excision generated deletion strains that differ from the wild type parental strain only by the absence of the target gene and the presence of a single FRT site. The integration of each nourseothricin cassette and subsequent excision was confirmed by colony PCR, using primers that bind either upstream and inside the cassette (X-upstream-fw and NAT-rv, where X represents the target gene; Figure 1C; Supplementary Table 4) or upstream and downstream of the deleted open reading frames (X-upstream-fw and X-downstream-rv; Figure 1D; Supplementary Table 4). As a

control, the same PCR reactions were performed with genomic DNA from the parental strain (Figure 1B), where all five target genes were intact, according to the published *C. glabrata* genome at the *Candida* Genome Database (Skrzypek, Binkley *et al*. 2017). Gene deletion experiments resulted in five different single mutants (*ato1*Δ, *ato2*Δ, *ato3*Δ, *fps1*Δ, and *fps2*Δ), two double (*ato1*Δ*ato2*Δ and *fps1*Δ*fps2*Δ), one triple (*ato1*Δ*ato2*Δ*ato3*Δ) and one quintuple (*ato1*Δ*ato2*Δ*ato3*Δ*fps1*Δ*fps2*Δ) mutants (Table 1).

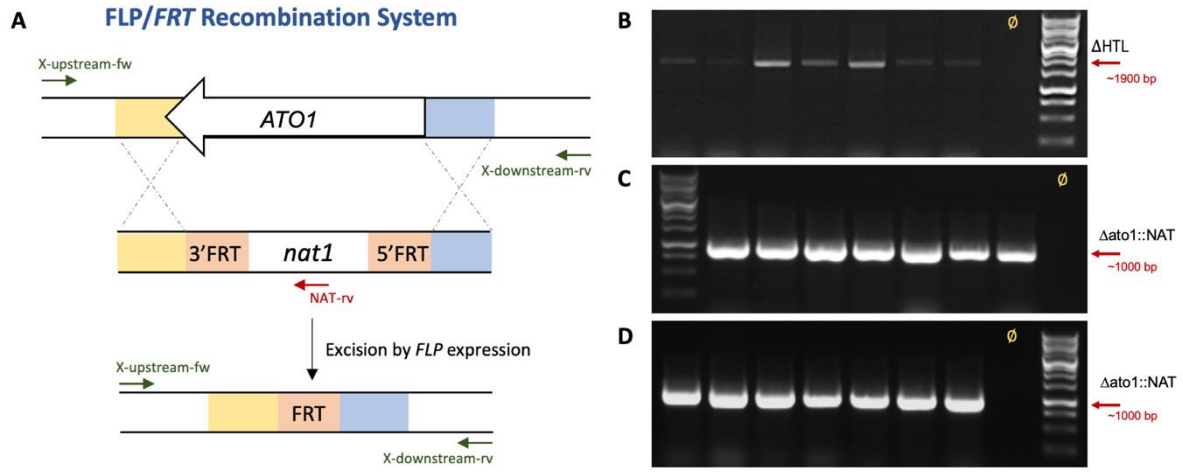

Figure 1. Schematic representation of the *SAT1* flipper method and diagnostic tests for the verification of correct cassette integration and deletion. A. Integration of nourseothricin (*nat1*) cassette by homologous recombination (regions of homology are approximately 100 bp long) and subsequent excision induced by the expression of *FLP*. **B.** Colony PCR reactions were performed on the genomic DNA of the parental strain as a control using X-upstream-fw and X-downstream-rv primers, where X represents the target gene (Supplementary Table 4). Each primer binds approximately 500 bp upstream or downstream of the target gene. **C.** Colony PCR reactions were performed on seven nurseothricin-resistant colonies obtained after transformation with the *nat1* cassette, using X-upstream-fw and NAT-rv primers (Supplementary Table 4). NAT-rv primer binds approximately 500 bp downstream of the start of the cassette. **D.** Colony PCR reactions were performed on seven hygromycin-resistant colonies obtained after transformation with the *FLP* expression plasmid to confirm gene deletion, by using X-upstream-fw and X-downstream-rv primers.

Lack of genes encoding putative acetate transporters leads to a diminished uptake of acetate by the mutants

C. glabrata mutant cells were also tested regarding their ability to transport radiolabeled acetic acid (Figure 2). The data show that a single deletion of any of the tested genes is sufficient to reduce the ability of the cells to transport acetate (statistically significant for all except the Δ*fps1*Δ strain; Figure 2). This data supports the hypothesis that these genes encode putative plasma membrane transporters and channels that mediate the transport of acetic acid.

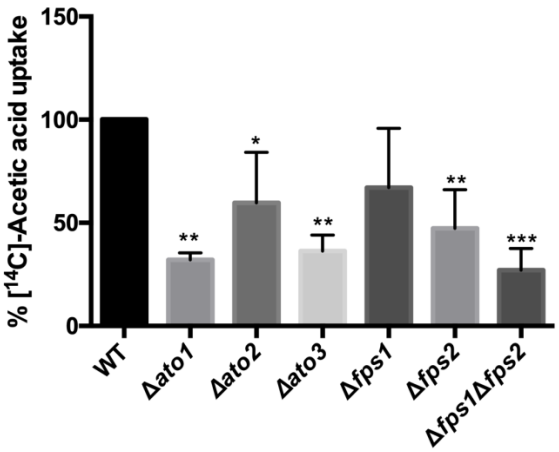

Figure 2. Transport assays support Ato and Fps function as acetate transporters. Percentage of 1 mM ^{14}C-acetic acid uptake, at pH 5.0, normalized to the velocity of transport of *C. glabrata* Δ*HTL* as 100%. Cells were cultivated in Synthetic Complete (SC) medium with 2% glucose until mid-exponential growth, washed and transferred for another 6 h growth in SC supplemented with 0.5% acetic acid, pH 5.0. Error bars show standard deviations. The data shown are mean values of triplicates of at least three independent experiments and significance was determined using two-way ANOVA with Tukey's multiple comparison test. *$p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$ indicate statistically significant results.

Heterologous expression of *C. glabrata* acetate transporters and channels in *S. cerevisiae* IMX1000 restore growth on acetic acid

To further determine if the function of the Ato proteins is conserved in *C. glabrata*, we examined the ability of Cg*ATO1*, Cg*ATO2*, Cg*ATO3*, Cg*FPS1*, and Cg*FPS2* to rescue the *S. cerevisiae* IMX1000 growth defect on acetic acid. This strain is deleted in 25 membrane-protein-encoding genes, including all the transporters known to mediate acetic acid uptake, namely Sc*ATO1*, Sc*ATO2*, Sc*ATO3*, and Sc*FPS1* (Mans, Hassing *et al*. 2017). As a control, we used the well-characterized acetate transporter gene Sc*ATO1* (Paiva, Devaux *et al*. 2004). Each gene was independently expressed in the IMX1000 strain in Synthetic Defined (SD) medium supplemented with 0.5% acetic acid. The pH of the medium was adjusted to 6.0, in order to guarantee that a high percentage of dissociated acid (acetic acid pKa=4.76) is present and enter the cell by a mediated transport mechanism. The obtained results show that the expression of each gene was sufficient to restore growth with acetate, suggesting that they play a role in acetate uptake (Figure 3).

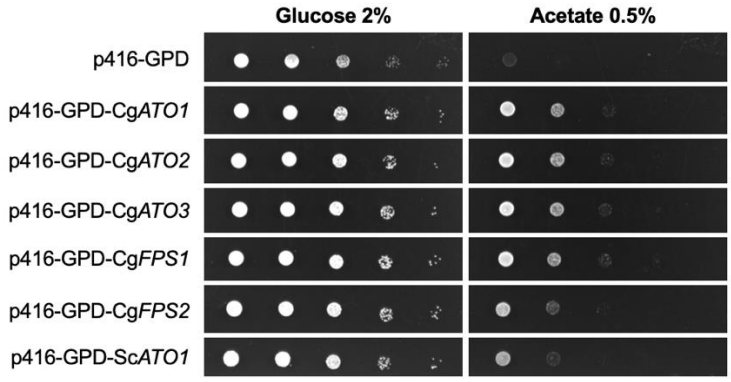

Figure 3. *S. cerevisiae* IMX1000 mutants heterologously expressing *C. glabrata* Ato transporters or Fps channels grow better on acetic acid. Cg*ATO1*, Cg*ATO2*, Cg*ATO3*, Cg*FPS1*, and Cg*FPS2* were independently cloned into the p416-GPD expression vector (Supplementary Table 3) and used to transform the IMX1000 strain. Serial dilutions were spotted on Synthetic Defined (SD) solid medium containing either glucose (2% w/v, pH 6.0) or acetate (0.5% v/v, pH 6.0) and supplemented with the required amino acids for growth. Pictures were taken after 9 days of incubation at 18ºC. Experiments were performed in triplicate, showing consistent results among all the independent assays.

To study the expression and subcellular localization of these proteins, we also used *S. cerevisiae* IMX1000 cells transformed with plasmids harboring genetic fusions of the transporter-encoding genes with fluorescent tags. The coding sequence of the fluorescent mCherry tag was cloned at the 3' end of each gene and introduced into the pBC6 expression vector, which contains the constitutive and strong *TEF* promoter. We were able to test only two of the transporters, CgAto1 and CgFps1, due to technical issues. Confocal microscopy revealed that both CgAto1-mCherry and CgFps1-mCherry localize to the plasma membrane (Figure 4). Although the mCherry signal was found predominantly at the cell surface for both fusion proteins (Figure 4), some fluorescence was also seen associated with intracellular structures in both cases. These structures resemble the endoplasmic reticulum, suggesting that the overexpression of these proteins, driven by the *TEF* strong promoter, may result in some ER retention. In contrast to CgAto1-mCherry, the CgFps1-mCherry signal was not uniformly distributed over the cell surface but rather appeared in patches, as previously reported for Sc*FPS1* (Figure 4) (Tamas, Luyten *et al*. 1999).

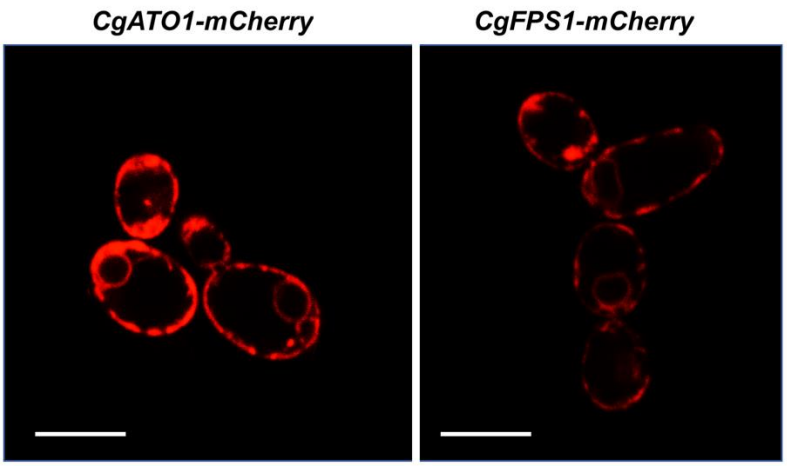

Figure 4. *C. glabrata* Ato1 transporter and Fps1 channel localize to the plasma membrane. The *S. cerevisiae* IMX1000 strain was transformed with pBC6 plasmids containing *CgATO1* (left) or *CgFPS1* (right), both tagged at their 3' end with the fluorescent marker mCherry (Supplementary Table 3). Representative images of a series taken with a Zeiss LSM 780 confocal microscope equipped with a 63 x NA1.4 oil immersion lens. Scale bars, 5 µm.

Relative expression of *C. glabrata* acetate transporters and channels during phagocytosis by human monocyte-derived macrophages

C. glabrata is particularly well-adapted to the intracellular environment of phagocytes (Kaur, Ma *et al*. 2007, Rai, Balusu *et al*. 2012, Fukuda, Tsai *et al*. 2013), being able to survive and replicate for extended periods of time within these cells (Seider, Gerwien *et al*. 2014, Duggan, Essig *et al*. 2015). The upregulation of Cg*ATO1* following phagocytosis has been supported by independent studies either in macrophages (Kaur, Ma *et al*. 2007) or neutrophils (Fukuda, Tsai *et al*. 2013). This transporter has also been shown to influence phagocytic uptake and killing of *C. glabrata* (Mota, Alves *et al*. 2015). We investigated the expression profile of *C. glabrata ATO1*, *ATO2*, *ATO3*, *FPS1*, and *FPS2* after phagocytosis by human monocyte-derived macrophages. *C. glabrata* cells were challenged with these phagocytes and retrieved at several time points over a period of seven days (Figure 5). As a control, we used yeasts from a log-phase YPD culture. Early after engulfment (6 h), we observed an upregulation of all investigated genes, with the exception of *CgATO3* (Figure 5). In particular, the expression of Cg*ATO2* was induced about 8-fold, a level which was maintained over time (Figure 5). Cg*ATO1* was the most upregulated gene over time (Figure 5), supporting previous reports (Kaur, Ma *et al*. 2007, Fukuda, Tsai *et al*. 2013). On the other hand, the expression of Cg*FPS2* decreased over time, recahing about 16-fold downregulation at days 4 and 7 (Figure 5). Interestingly, the expression profile of the cells following phagocytosis was very similar with the one observed in cells growing in a long-term, stationary YPD culture (stars, Figure 5), but different from the one found when cells were growing in a log-phase YPD

culture (inverted triangles, Figure 5). This suggests that *C. glabrata* cells within macrophages might be starving just like those experiencing a long stationary phase.

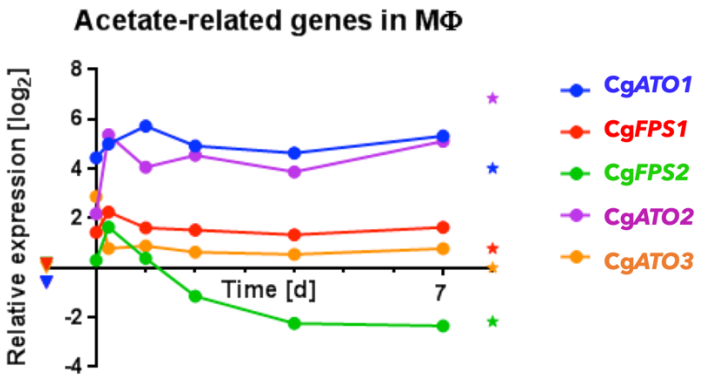

Figure 5. Relative expression of *C. glabrata* acetate transporters and channels during phagocytosis by human monocyte-derived macrophages. Relative expression of Cg*ATO1*, Cg*ATO2*, Cg*ATO3*, Cg*FPS1*, and Cg*FPS2* in response to phagocytosis by macrophages after 0, 6, 24 (1 day), 48 (2 days), 96 (4 days), and 168 h (7 days) based on microarray data. Triangles and stars indicate the expression of genes in *C. glabrata* cells when growing in log-phase YPD (left side, ▼) and long-term YPD (right side, ★), respectively.

Acetate transporter mutants show higher survival after long-term macrophage experiment

The triple and quintuple acetate transporter mutants of *C. glabrata* were subjected to phagocytosis by human monocyte-derived macrophages for 7 days, monitoring their survival at the time points of 3 hours, 1 day, 4 days, and 7 days. All strains showed similar uptake rate and killing rate during the first hours (data not shown), but a significant better survival of both mutants after 7 days (Figure 6). These results point to a relevant role of the acetate transporters during long-term phagocytosis.

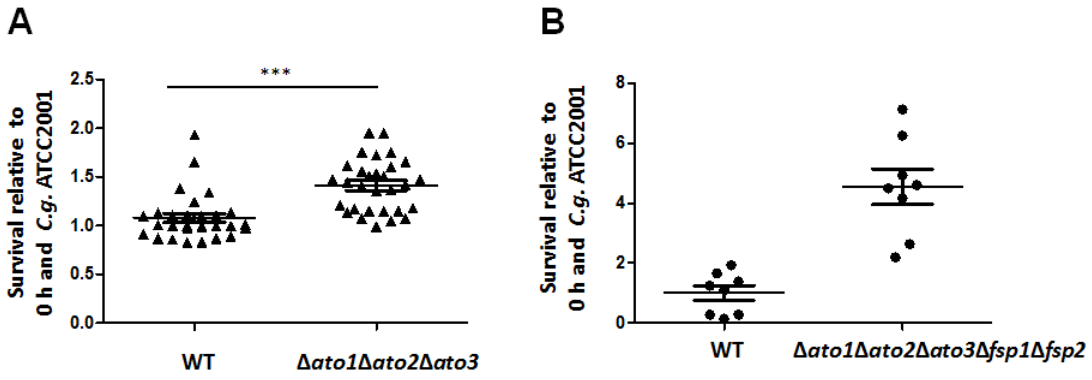

Figure 6. Acetate transporter mutants show increased survival in a long-term macrophage experiment. The triple *ato*Δ mutant (**A**) and quintuple *ato*Δ*fsp*Δ mutant (**B**) survive better than the wild type after 7 days inside of phagocytes. Mean ± SD, n=3 with 3 independent donors in 3 independent experiments, each point represents an independent survival event. Statistically significantly different values (unpaired,

two-tailed Student's t-test on log-transformed ratios) are indicated by asterisks as follows: ***, p ≤ 0.001.

Discussion

Acetate is present in different human niches: Produced by intestinal microbiota in the intestine (Yamaguchi, Sonoyama *et al*. 2005), on the vaginal mucosa (Amabebe and Anumba 2018), and within the phagosome (Lorenz, Bender *et al*. 2004, Danhof, Vylkova *et al*. 2016). It is known that one of the main pathogenicity mechanisms of the opportunistic pathogen *C. glabrata* is its ability to persist and replicate within the phagosome (Seider, Heyken *et al*. 2010, Seider, Gerwien *et al*. 2014, Kasper, Seider *et al*. 2015). In this study, the possible role of acetate transporters and channels in the persistence and growth of *C. glabrata* during phagocytosis was investigated. First, it was shown that the putative acetate transporter (*ATO*) and channel (*FSP*) genes are involved in the uptake of acetate from the medium, and that they can restore growth of an *S. cerevisiae* strain which lacks these transporters on acetate-containing medium. Furthermore only two genes of the *ATO* gene cluster were found to be upregulated during a long-term phagocytosis experiment, in agreement with previous observations during short-term interactions with phagocytes (Kaur, Ma *et al*. 2007, Fukuda, Tsai *et al*. 2013). This pattern of upregulation is similar to late stationary phase after most nutrients are depleted, and indicates a condition of starvation within the phagosome. In this scenario, the transporters would take up the acetate present in the phagosome and use it as carbon source, supporting growth and persistence of the fungus. However, contrary to this assumption, we observed that triple and quintuple mutants, lacking all *ATO* or all *ATO* and *FSP* genes, survived even better during long-term residence in macrophages (7 days). Mota *et al*. previously observed that acetate-grown wild type *C. glabrata* cells are better phagocytosed and killed than those grown in glucose medium (Mota, Alves *et al*. 2015). In addition, in the presence of acetate the mutant *ato1*Δ was much more refractory to killing when compared to wild type cells (Mota *et al*., 2015). That study concluded that the presence of acetic acid is not an advantage for fungal cells confronted with macrophages. These results also agree with recent observations that show a higher mannan exposure upon exposure to acetate, which may lead to increased uptake by phagocytes (Keppler-Ross, Douglas *et al*. 2010) and a lower oxidative stress resistance (Chew, Ho *et al*. 2019). Taken together, those previous observations and our new results indicate that metabolism of acetate within the phagosome could be disadvantageous for *C. glabrata* during long-term phagocytosis. Especially since removing all putative acetate transporters and channels led to a significantly increased survival, these genes could be classified as potential antivirulence genes of *C. glabrata* (Siscar-Lewin, Hube *et al*. 2019).

It is known that deletion mutants can trigger compensatory mechanisms (Schwarzmuller, Ma *et al*. 2014) that could be responsible for the overall increased survival of the multiple deletion mutants, such as the upregulation of other carboxylic acid, fatty acid or amino acid transporters, which are known to be used by phagocytosed fungi (Lorenz, Bender *et al*. 2004, Seider, Heyken *et al*. 2010, Seider, Gerwien *et al*. 2014, Kasper, Seider *et al*. 2015). For intance, it has been recently proposed that the most pathogenic *Candida* species, *C. albicans*, responds differently to each alternative carbon source, which it encounters during infection (carboxylic acids such as lactate, amino acids, and *N*-acetylglucosamine), inducing a specific pattern of protection from host-relevant stressors (Williams and Lorenz 2020). Exposure to these nutrients alters interactions with immune cells, likely as a result of significant changes in the fungal cell wall (Williams and Lorenz 2020). Analogously to *C. albicans*, increased uptake and metabolism of other alternative carbon sources in the acetate-transporters/channels mutants of *C. glabrata* might therefore be beneficial for the fungus to survive long-term residence within macrophages.

Material and methods

Yeast strains, plasmids, primers and growth conditions

The yeast strains, plasmids and primers used in this study are listed as Supplementary data. All *C. glabrata* mutant strains (Supplementary Table 1) were generated from the *C. glabrata* Δ*HTL* reference strain, derived from the ATC2001 strain with deletions in the histidine (*HIS3*), tryptophan (*TRP1*), and leucine (*LEU2*) loci (Jacobsen, Brunke *et al*. 2010). Cultures were routinely grown either in YPD (1% yeast extract, 2% peptone and 2% glucose), synthetic complete (SC) medium (0.67% yeast nitrogen base with ammonium sulfate, 2% glucose or 0.5% acetic acid, supplemented with a complete amino acid mixture - CSM from Formedium) or synthetic defined (SD) medium (0.67% yeast nitrogen base with ammonium sulfate, 2% glucose or 0.5% acetic acid, supplemented with the appropriate mixture of amino acids) at 37ºC. Both SC and SD media were buffered either at pH 5.0 or 6.0, as specified for each experiment. For solid YPD, SC or SD media, 1.5% agar was added.

Heterologous expression of *C. glabrata* transporters and channels in *S. cerevisiae*

C. glabrata *ATO1* (CAGL0M03465g), *ATO2* (CAGL0L07766g), *ATO3* (CAGL0A03212g), *FPS1* (CAGL0C03267g), and *FPS2* (CAGL0E03894g) were cloned into the p416-GPD vector (Mumberg, Müller *et al*. 1995) by homologous recombination using the *Escherichia coli* strain DH5α (Jacobus and Gross 2015). Each gene was amplified by PCR from about 50 ng of genomic DNA from the *C. glabrata* Δ*HTL* strain per 50 µL reaction volume. Yeast specific primers were designed based on the available

C. glabrata genome at CGD (Skrzypek, Binkley *et al*. 2017) and are listed in Supplementary Table 4 (p416-GPD-x-fw and p416-GPD-x-rv, where x represents the target gene). PCRs were carried out using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) following the manufacturer's instructions. The thermocycler setting consisted of one step of 98ºC for 30 sec, 30 cycles of 98ºC for 30 sec, 56ºC for 30 sec and 72ºC for 1 min, and one step of 72ºC for 10 min. The p416-GPD vector was digested with FastDigest *EcoRI* (Thermo Fisher Scientific) for 1 h in the appropriate buffer, following the manufacturer's instructions. Competent *E. coli* cells were transformed by the heat shock method (Maniatis, Fritsch *et al*. 1998). Briefly, each PCR fragment was mixed with the linear vector in a 10 µL volume of pure water. Then this volume was transferred into a microcentrifuge tube containing 50 µL of thawed competent cells and the mixture was incubated for 20 min on ice. Heat shock was carried out by placing the tubes in a water bath for 45 s at 42°C. Immediately after the heat shock, cells were placed on ice for 5 min. Then the thermal-shocked cells were recover in LB medium at 37°C for 1 h. After that time, cells were plated on solid LB medium containing ampicillin as selective antibiotic. Cells grew overnight at 37°C. To test for positive clones, cells from 8 randomly chosen colonies were tested by colony PCR, as previously described, using primers p416-GPD-X-fw and p416-GPD-X-rv (Supplementary Table 4). Plasmid DNA was purified from selected positive clones to confirm the correct insertion of the fragment into the vector by sequencing (Eurofins Genomics GmbH, Germany).

Fluorescent microscopy

C. glabrata cells were grown overnight for fluorescent microscopy using SD-glucose medium supplemented with histidine and leucine as described above. A volume of 1 mL of each cell culture was harvested, concentrated and manually immobilized on slides. Cells were immediately visualized under a Zeiss LSM 780 inverted confocal microscope using a 63x oil immersion objective and the 488 nm argon laser. Images were processed using the Zeiss ZEN 2010 software (Carl Zeiss, Jena, Germany).

Mutant generation

Gene deletions were performed in the Δ*HTL* strain by homologous recombination, using the *SAT1* flipper method (Shen, Guo *et al*. 2005). Briefly, a cassette containing the *C. albicans NAT* (Ca*NAT1*) gene, which confers nourseothricin resistance, flanked by FLP recombinase recognition target (FRT) sites, was amplified by PCR from the pYC44 plasmid (Supplementary Table 2) (Yanez-Carrillo, Orta-Zavalza *et al*. 2015), using forward and reverse primers that included 100 nucleotides noncoding sequence

upstream or downstream of each selected gene (Supplementary Table 3). The PCR reaction was performed using Ex Taq DNA Polymerase (Takara) according to the manufacturer's protocol. The thermocycler setting consisted of one step of 98ºC for 30 sec, 30 cycles of 98ºC for 30 sec, 60ºC for 30 sec and 72ºC for 2 min, and one step of 72ºC for 10 min. The correct size of each fragment was confirmed by electrophoresis. *C. glabrata* Δ*HTL* cells from a YPD preculture were diluted into 50 mL fresh YPD medium to an optical density at 600 nm (OD_{600nm}) of approximately 0.2 and grown at 37ºC for 3-4 h to an OD_{600nm} of approximately 1.5. Cells were then collected by centrifugation at 3000 *g* for 5 min, washed two times with 50 mL of sterile water and finally resuspended in 8 mL of sterile water. After addition of 1 mL of 10× TE (100 mM Tris-HCl, 10 mM EDTA, pH 7.5) and 1 mL of 1 M lithium acetate (pH 7.5), the suspension was incubated in a rotary shaker at 200 rpm for 30 min at 37ºC. Then, 250 µL of 1 M dithiothreitol were added to the cell culture and incubated for 1 h at 37ºC with shaking. After addition of 40 mL of sterile water the cells were centrifuged at 3000 *g* for 5 min, and kept on ice until the electroporation step. Cells were washed sequentially two times in 25 mL of ice-cold water, one time in 5 mL of ice-cold 1 M sorbitol and resuspended in 500 µL of ice-cold 1 M sorbitol. For each transformation, 45 µL of electrocompetent cells and 5 µL of the amplified NAT cassette (approximately 1 µg) were mixed and electroporated at 1.5 kV; 200 Ω; 25 µF. Recovery was carried out by adding 2 mL of YPD and incubating at 37ºC for 4 h with shaking. Cells were then harvested at 3000 *g* for 5 min, resuspended in 100 µL of sterile water and plated on YPD containing 200 $\mu g \cdot mL^{-1}$ of nourseothricin (clonNAT; Jena Bioscience GmbH, Germany). Resistant colonies were picked after 1-2 days of growth and confirmed by colony PCR analysis, using x-upstream-fw and NAT-rv primers, where x represents the target gene (Supplementary Table 3). The PCR reaction was performed using a Taq DNA Polymerase. The thermocycler setting consisted of one step of 98ºC for 10 min, 30 cycles of 98ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min, and one step of 72ºC for 10 min. Positive confirmed transformants were streaked on YPD plates containing 200 $\mu g \cdot mL^{-1}$ of clonNAT for further use. To create the multiple mutants, the *NAT* cassette was removed by transforming the clonNAT resistant mutants with the pLS10 vector expressing the *FLP1* recombinase (Supplementary Table 3). Strains containing the plasmid were selected on YPD plates containing 300 $\mu g \cdot mL^{-1}$ of hygromycin B (HYG; Invitrogen, CA, USA). Resistant colonies were picked up after 2 days of growth and confirmed by colony PCR analysis using primers x-upstream-fw and x-downstream-rv (Supplementary Table 3), as previously described.

Transport assays

Measurement of transport activity in *C. glabrata* strains was performed as previously

described (Sa-Pessoa, Paiva *et al*. 2013). Briefly, *C. glabrata* cells were incubated in 50 mL of SC medium supplemented with 2% glucose (pH 5.0) at 37ºC, 200 rpm, until $OD_{600nm} \approx 0.5$. Then cells were harvested by centrifugation, washed twice in sterile water, resuspended in SC medium supplemented with 0.5% acetic acid, pH 5.0, and incubated again for 6 hours. Cells were then washed with sterile water and resuspended in 0.1 M potassium phosphate buffer, pH 5.0, to a final concentration of about 5-15 $mg \cdot mL^{-1}$ dry weight. Then, 30 µL of each yeast cell suspension were added to 60 µL of phosphate buffer in a microtube and after 2 min of incubation at 30ºC, the reaction was started by the addition of 10 µL of an aqueous solution of [1-^{14}C]acetate, sodium salt (10 mM, pH 6.0; specific activity of 2000 d.p.m./nmol) purchased from Perkin Elmer. The reaction was stopped by the addition of ice-cold 100 mM non-labelled acid at pH 6.0 after 15 seconds. The reaction mixtures were centrifuged at 4ºC for 5 min at 16,000 g, the pellet was resuspended by vortexing in 1 mL of ice-cold water, centrifuged again and finally resuspended in 1 mL of scintillation liquid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies). Radioactivity was measured in a Packard Tri-Carb 2200CA liquid scintillation spectrophotometer with d.p.m. correction.

Isolation and differentiation of human monocyte-derived macrophages (hMDMs)
Blood was obtained from healthy human volunteers with written informed consent according to the declaration of Helsinki. The blood donation protocol and use of blood for this study were approved by the Jena institutional ethics committee (Ethik-Kommission des Universitätsklinikums Jena, Permission No 2207–01/08). Human peripheral blood mononuclear cells (PBMCs) from buffy coats donated by healthy volunteers were separated through Lymphocytes Separation Media (Capricorn Scientific) in Leucosep™ tubes (Greiner Bio-One) by density centrifugation. Magnetically labeled CD14 positive monocytes were selected by automated cell sorting (autoMACs; MiltenyiBiotec). To differentiate PBMC into human monocyte-derived macrophages (hMDMs), 1.7×10^7 cells were seeded into 175 cm² cell culture flasks in RPMI1640 media with L-glutamine (Thermo Fisher Scientific) containing 10 % heat-inactivated fetal bovine serum (FBS; Bio&SELL) and 50 $ng \cdot mL^{-1}$ recombinant human macrophage colony-stimulating factor M-CSF (ImmunoTools). Cells were incubated for five days at 37°C and 5 % CO_2 until the medium was exchanged. After another two days, adherent hMDMs were detached with 50 mM EDTA in PBS and seeded in 96-well plates (4×10^4 hMDMs/well) for survival-assay, in 12-well-plates (4×10^5 hMDMs/well) for intracellular replication assay with 100 $U \cdot mL^{-1}$ γ-INF, and 24-well-plates for long-term experiment (1.5×10^5 hMDMs/well) without γ-INF. Prior to macrophage infection, medium was exchanged to serum free-RPMI medium and 100 $U \cdot mL^{-1}$ γ-INF. For long-term

experiment, the medium was exchanged to RPMI1640 containing 10 % human serum (Bio&Sell 1: B&S Humanserum sterilised AB Male, Lot: BS.15472.5).

Long-term phagocytosis-survival assay

The long-term experiment was perform in 24-well-plates were the cells were infected with a MOI of 1 and incubated for one week at 37°C and 5% CO_2. After 3 h of coincubation, cells were washed with PBS and the medium was exchanged to RPMI 1640 containing 10 % human serum. For the 3 h time point both supernatant and lysate were plated. From the day 1 to day 7 times points, one third of the medium was exchanged every day by fresh RPMI 1640 with 10% human serum. At 3 hours, 1 day, 4 days, and 7 days the lysate of 4 different wells was diluted accordingly and 200 CFU were plated on 8 YPD agar plates, which were afterwards incubated for 48 h at 37°C.

Statistical analysis

All the results were obtained from at least three biological replicates (indicated in figure legends). Mean and standard deviations of these replicates are shown. MDMs used in experiments were isolated from at least three different donors (see figure legends).

Supplementary Table 1. *C. glabrata* strains used and generated in this study.

Name	Relevant genotype	Parental strain	Origin
Δ*HTL*	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT	ATCC 2001	(Schwarzmuller, Ma *et al*. 2014)
CGRA01	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::natNT2	Δ*HTL*	This study
CGRA04	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT	CGRA01	This study
CGRA07	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato2*Δ::natNT2	Δ*HTL*	This study
CGRA10	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato2*Δ::FRT	CGRA07	This study
CGRA13	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato3*Δ::natNT2	Δ*HTL*	This study
CGRA16	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato3*Δ::FRT	CGRA13	This study
CGRA19	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *fps1*Δ::natNT2	Δ*HTL*	This study
CGRA22	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *fps1*Δ::FRT	CGRA19	This study
CGRA25	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *fps2*Δ::natNT2	Δ*HTL*	This study
CGRA28	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT	CGRA25	This study

	*fps2*Δ::FRT		
CGRA31	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *fps1*Δ::FRT *fps2*Δ::natNT2	CGRA22	This study
CGRA34	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *fps1*Δ::FRT *fps2*Δ::FRT	CGRA31	This study
CGRA37	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::natNT2	CGRA04	This study
CGRA40	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::FRT	CGRA37	This study
CGRA43	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::FRT *ato3*Δ::natNT2	CGRA40	This study
CGRA46	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::FRT *ato3*Δ::FRT	CGRA43	This study
CGRA49	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::FRT *ato3*Δ::FRT *fps1*Δ::natNT2	CGRA46	This study
CGRA52	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::FRT *atot3*Δ::FRT *fps1*Δ::FRT	CGRA49	This study
CGRA55	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *atot1*Δ::FRT *ato2*Δ::FRT *ato3*Δ::FRT *fps1*Δ::FRT *fps2*Δ::natNT2	CGRA52	This study
CGRA58	ATCC 2001 *his3*Δ::FRT *leu2*Δ::FRT *trp1*Δ::FRT *ato1*Δ::FRT *ato2*Δ::FRT *ato3*Δ::FRT *fps1*Δ::FRT *fps2*Δ::FRT	CGRA55	This study
CGRA61	Δ*HTL* + pBC6-mcherry-Cg*ATO1*	Δ*HTL*	This study
CGRA63	Δ*HTL* + pBC6-mcherry-Cg*ATO2*	Δ*HTL*	This study
CGRA66	Δ*HTL* + pBC6-mcherry-Cg*ATO3*	Δ*HTL*	This study
CGRA69	Δ*HTL* + pBC6-mcherry-Cg*FPS1*	Δ*HTL*	This study
CGRA72	Δ*HTL* + pBC6-mcherry-Cg*FPS2*	Δ*HTL*	This study

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

Name	Relevant genotype	Parental strain	Origin
IMX1000	*MATa ura3-52 trp1-289 leu2-3112 his3*Δ can1::cas9-natNT2 *mch1*Δ *mch2*Δ *mch5*Δ *aqy1*Δ *itr1*Δ *pdr12*Δ *mch3*Δ *mch4*Δ *yil166c*Δ *hxt1*Δ *jen1*Δ *ady2*Δ *aqr1*Δ *thi73*Δ *fps1*Δ *aqy2*Δ *yll053c*Δ *ato2*Δ *ato3*Δ *aqy3*Δ *tpo2*Δ *yro2*Δ *azr1*Δ *yhl008c*Δ *tpo3*Δ	CEN.PK113-7D	(Mans, Hassing *et al*. 2017)
SCRA01	IMX1000 + p416-Cg*ATO1*	IMX1000	This study
SCRA04	IMX1000 + p416-Cg*ATO2*	IMX1000	This study
SCRA07	IMX1000 + p416-Cg*ATO3*	IMX1000	This study
SCRA10	IMX1000 + p416-Cg*FPS1*	IMX1000	This study
SCRA13	IMX1000 + p416-Cg*FPS2*	IMX1000	This study

SCRA16	IMX1000+ p416-ScATO1	IMX1000	This study

Supplementary Table 3. Plasmids used and generated in this study.

Name	Description	Origin
pYC44	Plasmid containing CaNAT1 flanked by FRT sites	(Yanez-Carrillo, Orta-Zavalza *et al*. 2015)
pLS10	Plasmid expressing FLP1 recombinase (HYG^R)	Van Dijck Lab
p416-GPD	Yeast expression vector containing GPD promoter	(Mumberg, Müller *et al*. 1995)
pBC6	Yeast expression vector containing TEF promoter	Van Dijck Lab
pYC42	Recipient mCherry replicative vector	(Yanez-Carrillo, Orta-Zavalza *et al*. 2015)
pYC55	Recipient YFP replicative vector	(Yanez-Carrillo, Orta-Zavalza *et al*. 2015)
p416-CgATO1	Plasmid expressing Cg-*ATO1* from GPD promoter	This study
p416-CgATO2	Plasmid expressing Cg-*ATO2* from GPD promoter	This study
p416-CgATO3	Plasmid expressing Cg-*ATO3* from GPD promoter	This study
p416-CgFPS1	Plasmid expressing Cg-*FPS1* from GPD promoter	This study
p416-CgFPS2	Plasmid expressing Cg-*FPS2* from GPD promoter	This study
pBC6-mcherry-CgATO1	Plasmid expressing N-tagged Cg*ATO1* with mCherry	This study
pBC6-YFP-CgATO1	Plasmid expressing N-tagged Cg*ATO1* with YFP	This study
pBC6-mcherry-CgATO2	Plasmid expressing N-tagged Cg*ATO2* with mCherry	This study
pBC6-YFP-CgATO2	Plasmid expressing N-tagged Cg*ATO2* with FP	This study
pBC6-mcherry-CgATO3	Plasmid expressing N-tagged Cg*ATO3* with mCherry	This study
pBC6-YFP-CgATO3	Plasmid expressing N-tagged Cg*ATO3* with YFP	This study
pBC6-mcherry-CgFPS1	Plasmid expressing N-tagged Cg*FPS1* with mCherry	This study
pBC6-YFP-CgFPS1	Plasmid expressing N-tagged Cg*FPS1* with YFP	This study
pBC6-mcherry-CgFPS2	Plasmid expressing N-tagged Cg*FPS2* with mCherry	This study
pBC6-YFP-CgFPS2	Plasmid expressing N-tagged Cg*FPS2* with YFP	This study

Supplementary Table 4. Oligonucleotides used in this study.

Oligonucleotide name	Sequence
ato1-pYC44-fw	TTACTAGATATAAAAAAAAAAAGTTACCTTTTTTTTTTTATTGAACGTACACTGCA CATAATTAAA TTGTTTCAAAATTAAGTTCCTACACAAATTTCAGgctctagaactagtggatcc
ato1-pYC44-rv	GACTAGCACGACATTTGTTATTATTATTGTTAGTGTTTTGTTTTATGGTTTAAATT GAAATTAAAA

	CCGTAAGATTTGAAATTGCAGAAGTTCAAAAAAAagggaacaaaagctggtacc
ato2-pYC44-fw	GACTTGATGTGGTTTCGATTGAAATTTACTTGTAGAACAGGTATAGATATTTGCT GGATAGGTAA TATACCAGTTGAATAATCAAAGAAACATAAAAAAAgctctagaactagtggatcc
ato2-pYC44-rv	AATTTCAGGAAAATTTAAATTGTTGCATACAGTAAAGTAGCGATTTATTTTTTTTC ATTATGTGCA TTTCTTAAGCTCTGCATCAGTTATTGTTAATATTagggaacaaaagctggtacc
ato3-pYC44-fw	AATTCCCTTGTGCACTCGTAAATTCAGTGTAGAACTCGTAAAAAATTGTGTACAA TATTACCGAT AAGCCACAAACACAATAACTAATCATATAAGTCACgctctagaactagtggatcc
ato3-pYC44-rv	CGTGAAGTAAAAATTATGCTATCTTGATGAGCGCAGAGTCTGGAGATGTGTAGA TTTTCGGAATA TAATTAAAAGGGACGAGATGAAGTTAGTTTTGGATagggaacaaaagctggtacc
fps1-pYC44-fw	CCGTCTAGCATTTTCTGGATATACATTATAAAAATACAATCAAAAATCAATAAAA AACAATCTAGA CTGATACTATTCTGATACTAAAGTATAACAAAAAgctctagaactagtggatcc
fps1-pYC44-rv	AATAATGCTAGAAAATGGGACGTGCTCTATCTCTTCCTTCCTGGGAACAGAAGC ACATTGAGGT ATACTATCCATGGGCATGACGATTCGTTCCGTTTATagggaacaaaagctggtacc
fps2-pYC44-fw	GACTATCTCAAAACAATACTAAAACAACTCGACAATCTACATTTTACTACTTTTCT TTGAAGAATA ATATACTACACTTTGAGACTCCAGCTTGACAAAGgctctagaactagtggatcc
fps2-pYC44-rv	GAATCATCAAATATTGCGTAAGTTATAAATATAACCTGGCGTTATGAGTGGAGT GGTAACAATTCA TGTAAACTCACATTTACTTTATGAGTAAGAGACCagggaacaaaagctggtacc
ato1-upstream-fw	GCTGTCTTTCGAATATCAACAACAC
ato1-dowstream-rv	GCTCTGTTTCAATTGTAGATCAATAGG
ato2-upstream-fw	GGTATTGAGTTCATCTCCGGTATAA
ato2-dowstream-rv	GTTACTGTGAGCGAAATATGAAGTAGTA
ato3-upstream-fw	CGATGCACAGAACGGATAAATTAAG
ato3-dowstream-rv	CTCGATGAACCCAGTGATGTC
fps1-upstream-fw	GTATATCGGATTACTTGTGCGCT
fps1-dowstream-rv	CTACTTGACGCTCGGACTG
fps2-upstream-fw	GTCCACGGACTTACCTATTCTG
fps2-dowstream-rv	CCTTGATGGACATAATAAGCTTCCT
NAT-rv	CGTCAAGACTGTCAAGGAGGG
p416-GPD-ATO1-fw	CTAGAACTAGTGGATCCCCCGGGCTGCAGGATGTCTGACAAAGATCAAGG
p416-GPD-ATO1-rv	TCGACGGTATCGATAAGCTTGATATCGAATTTAGTATAGGGTCTTTTCGTTT
p416-GPD-ATO2-fw	CTAGAACTAGTGGATCCCCCGGGCTGCAGGATGGTTTCTATTAGCTCCAG
p416-GPD-ATO2-rv	TCGACGGTATCGATAAGCTTGATATCGAATTCTAAAAAAACACCTTCTCGTTA
p416-GPD-ATO3-fw	CTAGAACTAGTGGATCCCCCGGGCTGCAGGATGAGCTCATCATCCTCACA
p416-GPD-ATO3-rv	TCGACGGTATCGATAAGCTTGATATCGAATTTTAGTTTGGCATCATGATGG
p416-GPD-FPS1-fw	CTAGAACTAGTGGATCCCCCGGGCTGCAGGATGTCTCATCAGCAAGGG
p416-GPD-FPS1-rv	TCGACGGTATCGATAAGCTTGATATCGAATTTCAGTTACTGTTCTTGGAAC

p416-GPD-FPS2-fw	CTAGAACTAGTGGATCCCCCGGGCTGCAGGATGGAATCTATTCATGATGCTATG
p416-GPD-FPS2-rv	TCGACGGTATCGATAAGCTTGATATCGAATTCTAATATGACACCTTCTCATCA
pBC6-BamHI-CgATO1-fw	TTTCTAGAACTAGTGGATCCACAATGTCTGACAAAGATCAA
pBC6-CgATO1-linker-rv	CCCGCCTCCAGATCCTCCGCCAGAGCCTCCCCCGTATAGGGTCTTTTCGTTTG
pBC6-BamHI-CgATO2-fw	TTTCTAGAACTAGTGGATCCACAATGAGCTCATCATCC
pBC6-CgATO2-linker-rv	CCCGCCTCCAGATCCTCCGCCAGAGCCTCCCCCAAAAAACACCTTCTCGTTAG
pBC6-BamHI-CgATO3-fw	TTTCTAGAACTAGTGGATCCACAATGAGCTCATCATCCTCACAG
pBC6-CgATO3-linker-rv	CCCGCCTCCAGATCCTCCGCCAGAGCCTCCCCCGTTTGGCATCATGATGGCTC
pBC6-BamHI-CgFPS1-fw	TTTCTAGAACTAGTGGATCCACAATGTCTCATCAGCAAGGG
pBC6-CgFPS1-linker-rv	CCCGCCTCCAGATCCTCCGCCAGAGCCTCCCCCGTTACTGTTCTTGGAACTGT
pBC6-BamHI-CgFPS2-fw	TTTCTAGAACTAGTGGATCCACAATGGAATCTATTCATGAT
pBC6-CgFPS2-linker-rv	CCCGCCTCCAGATCCTCCGCCAGAGCCTCCCCCATATGACACCTTCTCATCAG
D12-linker-mCherry-fw	GGAGGATCTGGAGGCGGGAGCGGCGGAGGTTCTATGGTGAGCAAGGGCGA
D13-mCherry-SacII-rv	AAACCCCGGGGCGGCCGCGGCTACTTGTACAGCTCGTCC
D14-linker-YFP-fw	GGAGGATCTGGAGGCGGGAGCGGCGGAGGTTCTatgAGTAAAGGAGAAGAAC
D15-YFP-SacII-rv	AAACCCCGGGGCGGCCGCGGCTATTTGTATAGTTCATCC

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7. Discussion

7.1 Evolution of fungal virulence: Learning from avirulence and antivirulence factors

Fungi, as any other living organism, need to complete their life cycle, which principally means to reproduce. To that end, they have developed a number of adaptations to acquire nutrients and protect themselves from threats in their environment. Commensal fungi of humans are a special case, as they have adapted to an environment with a relatively constant nutrient availability, temperature, and an absence of strong fluctuations in the environment (drastic changes in temperature, pH, toxic compounds and competition). During the evolution as commensals, these microbes have inactivated or lost genes that are not adaptive in the host environment, becoming more specialized to their host niches. These genetic events may have allowed commensal fungi to gain virulence potential, as the loss of these genes, the so-called antivirulence genes, also have a positive effect in the establishment and progression of an infection. Thus in conditions of host immunodeficiency and dysbiosis, these fungi can realize their virulence factors and become pathogenic, and are then called opportunistic pathogens. However, in normal conditions of immunocompetence these opportunistic pathogens are still kept in check by the host immune response and other microbiota, which prevents uncontrolled colonization, damage, and disease. Furthermore, under conditions of immunocompetence the host immunosurveillance, which co-evolved with these fungi, can recognize their virulence factors or activities and exploit them to its own benefit or initiate counter activities, keeping the fungi functionally avirulent. Nowadays, however, the number of immunocompromised hosts is increasing *e.g.* due to malignancies or by advanced medical interventions, and environmental and commensal fungi can more readily cause severe diseases by employing their specific virulence factors (Brown, Denning *et al*. 2012, Kohler, Hube *et al*. 2017).

The human host has evolved to allow fungal and other, especially bacterial commensals, to thrive on its mucosae, and positive effects of a stable mycobiome for the host have been reported (reviewed by Iliev and Leonardi 2017, Limon, Skalski *et al*. 2017, Lai, Tan *et al*. 2019). The IFN-1 mitochondrial-dependent immunomodulatory pathway is an explicit example of how the host detects and tolerates the presence of *Candida* species as commensals on vaginal epithelial cells, despite them being the most common causes of vulvovaginal candidiasis (Pekmezovic, Mogavero *et al*. 2019). Only upon epithelial

damage, the host activates its antifungal defense mechanisms in species-specific manner, depending on the level of damage caused by each *Candida* species (Pekmezovic, Hovhannisyan *et al*. 2021).

Since the degree of host damage defines how virulent a fungus is, high virulence would therefore only be adaptive when damage and disease are necessary in the fungus' life cycle, *e.g.* to spread to other hosts, as it is the case for many obligate pathogens. These pathogens, such as *Yersinia pestis* or *Mycobacterium tuberculosis* possess specific virulence factors which cause disease symptoms, because their survival of host counterattacks and their transmission depend on it (Du and Wang 2016, Ehrt, Schnappinger *et al*. 2018). In contrast, the majority of fungal opportunistic pathogens lack such highly specialized factors, since their survival is normally not linked to host damage and their transmissibility does not depend on such outcome (Casadevall and Pirofski 2019). Recently it has been suggested that many virulence traits of *C. albicans*, the most pathogenic *Candida* species, may have rather evolved to enable the fungus to spread by causing limited mucous membrane infections. These would occur mainly in infants, since they are the human population naturally susceptible to *C. albicans*: Thrush is common in children before developing anti-*Candida* adaptive immunity (Jennison 1977). Infected infants may spread the fungus to their immune-competent relatives and so maintain *Candida* as a commensal in a large population of human hosts. In this model, *C. albicans* would adjust its virulence determinants to act (in a limited fashion) against infants, accidentally enabling the fungus to eventually drive devastating invasive infections among the large immunocompromised populations nowadays (Kohler, Acosta-Zaldivar *et al*. 2020). The virulence traits of *C. albicans* would therefore be beneficial for the stable existence of the fungus among the human population. However, related commensal *Candida* species, like *C. dubliniensis*, do not show virulence attributes similar to *C. albicans,* and in fact seem to be losing virulence potential over evolutionary timescales (Moran, Coleman *et al*. 2012). This can be explained by a different strategy: In the trade-off between host damage and host response, they follow the route of less immune recognition and damage, while maintaining the ability to colonize new host niches. This may explain why the two very closely related commensal species, *C. albicans* and *C. dubliniensis* are evolving separately and differ in virulence – more virulent and more commensal, respectively, – and why the degree of virulence in both, commensal and environmental fungi, can change drastically within the same population upon changes in the host environment.

Unlike *C. dubliniensis, C. albicans* seems to have evolved toward increased virulence.

More virulent phenotypes may confer the ability to conquer new niches beyond the commensal environment, but as a result of host-pathogen co-evolution, the host also has "learnt" to recognize such virulence factors and counterattack to stop the invading pathogen. In that scenario, the fungal virulence factors become then avirulence factors, and the pathogen, therefore, may need to regain virulence potential by evolutionary attenuating immune recognition. In this way, pathogen and host would benefit, as the pathogen increases its chance to establish itself in new niches, but induces less damage in the host. One possible example for this is candidalysin in *Candida* species (Moyes, Wilson *et al*. 2016, Siscar-Lewin, Hube *et al*. 2019, Koenig, Hube *et al*. 2020). Despite being the main cause of damage by *C. albicans*, the intrinsic ability of synthetic versions of candidalysin from the different species to cause damage and epithelial immune response is actually more potent in the related, less pathogenic species *C. dubliniensis* and *C. tropicalis* (Jonathan Richardson, personal communication). In these species the damage potential and thus the immune reaction may be attenuated by reducing the expression of the genes coding for this toxin (*ECE1*) during infection. Potentially, *ECE1* orthologues in these species may have very specific functions and may only be expressed under very defined conditions. In contrast, in *C. albicans ECE1* shows the highest transcript levels, but the peptide itself seems to have less damage potential and immune reactivity – thus keeping its virulence and, importantly, its avirulence effects during infection lower. In agreement with this view, the precursor of candidalysin, containing Lys and Arg (KR) at the C-terminus is more toxic than the final mature toxin ending with Lys (K). The lower damage and immune trigger capacity may have resulted from the co-evolution of host and pathogen. A weaker toxin would not only prevent a fast, strong epithelial disruption, but also a strong and rapid immune reaction, which would be detrimental for the infection progression and for the host, since it would lead to a higher cell and tissue damage. These differences may boil down to a minor structural variation: while the candidalysin secondary structure is similar in all three species, the presence of a central proline in the two milder pathogens and its absence in *C. albicans* has been shown as an important reason for the differences in activity. In addition, the C-terminus of the *C. albicans* candidalysin is more sensitive to amino acid substitutions, which decreases its biological activity (epithelial damage, calcium influx and cytokine secretion) in contrast to *C. dubliniensis* or *C. tropicalis* candidalysins (Jonathan Richardson, personal communication).

Similar examples of such adaptations to escape immune surveillance have been reported in plant pathogenic fungi, where avirulent effectors can "regain" their virulence potential in a variety of manners, from mutations in their gene sequence, to changes in the regulation of their expression by epigenetic and epistatic mechanisms (Na and Gijzen

2016). For instance, certain strains of the hemibiotrophic fungi leaf tomato mold *Cladosporium fulvum* show mutations in avirulence genes that produce modified avirulent effectors. These are not recognized anymore by the receptors of resistant (to the avirulent form) plants, but still exert their (original) virulence traits. Therefore the plant's immune response, known as hyper-sensitive response (HR) (localized cell death at the site of infection, enforcements of plant cell walls, release of reactive oxygen species, and *de novo* production of antimicrobial compounds) is no longer activated, and its resistance is thereby lost – turning the fungal avirulence gene back into a virulence gene (Iida, van't Hof *et al*. 2015) (Figure 1). Thus, as a result of co-evolution, the pathogens evolved a counter-strategy against the host attack on the pathogen's virulence weapons, allowing the infection to progress again. Analogously, these events that have been accepted as important in plant pathogens are also at work in fungal pathogens of humans (Koenig, Muller *et al*. 2021). Those human pathogenic fungi, which are normally associated with their host as commensals, depend on viable hosts. These fungi may thus also prevent a possible self-damaging host response, similarly to biotrophic fungi, that would not only be detrimental for the host's health, but also for the pathogen's survival.

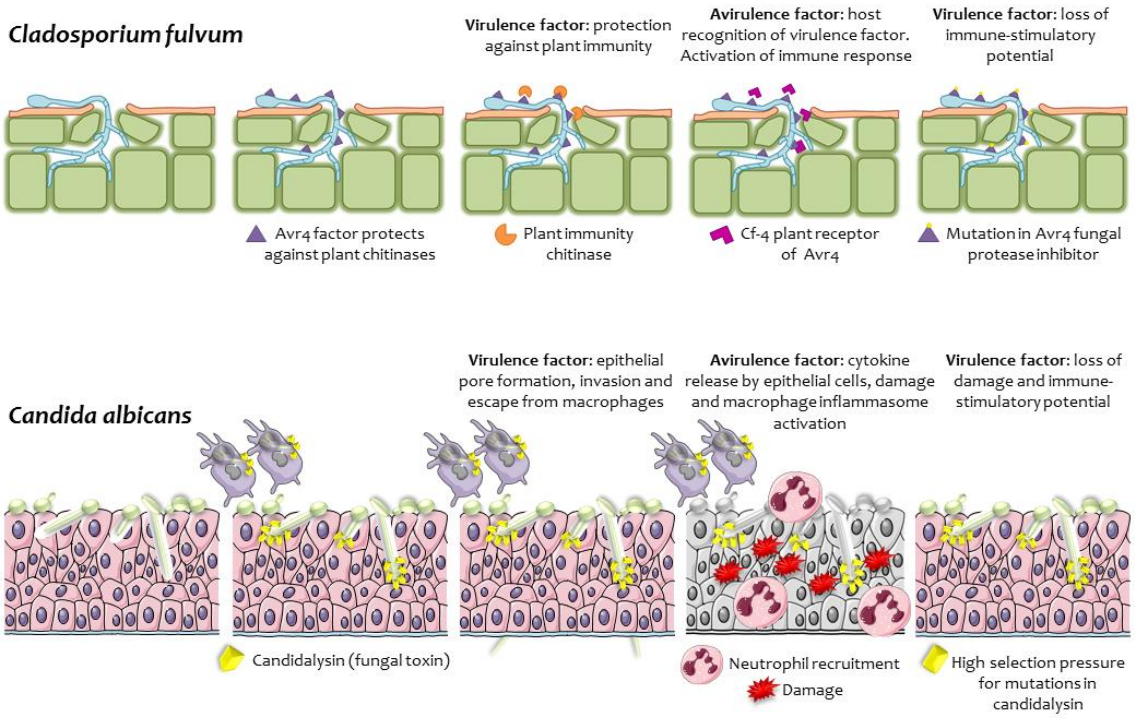

Figure 1. **Analogy of avirulence factor's evolution of two pathogenic fungi (plant and human) during host-pathogen interaction.** The upper scheme shows the co-evolution of the hemibiotrophic plant pathogen *Cladosporium fulvum* with its host tomato plant (*Lycopersicon* spp). The fungus possesses the counter-

defensive virulence factor Avr4 that protects the fungal cell walls against hydrolysis by plant chitinases (van Esse, Bolton *et al*. 2007), which form part of the plant's basal defense, making the host susceptible of infection. This factor is recognized by the plant receptor Cf-4 that activates plant immunity, rendering the plant resistant and the pathogen avirulent (Postma, Liebrand *et al*. 2016). As a further step of fungi counterattack, the factor Avr4 undergoes mutation and high selective pressure to regain virulence potential by preventing recognition Cf-4 and activation of plant immunity (Iida, van 't Hof *et al*. 2015). In the figure bellow the human pathogenic fungus *Candida albicans* produces the toxin candidalysin that allows the fungus to gain nutrients from host cells during invasion of host epithelium and to escape from macrophages, which form part of the first line of host immune defense. A certain threshold of candidalysin activates the epithelial "danger response" and the inflammasome in macrophages promoting the release of immunostimulatory cytokines and the activation of antifungal defense response by recruiting neutrophils to the site of infection (Moyes, Wilson *et al*. 2016, Kasper, Koenig *et al*. 2018). A recent study shows that *C. albicans*'s candidalysin is less damaging and immunostimulatory than the homologous synthetic versions in other related species likely due to the toxin's amino acid sequence (Jonathan Richardson, personal communication). Both scenarios represent how the emergence and evolution of a virulence factor is a trade-off between escaping detection and optimizing the virulence-related functions.

In both commensal and environmental fungal species many genes are identified as putative antivirulence genes whose loss or inactivation can increase the virulence potential of the fungus and may thereby confer an adaptive advantage during infection (Siscar-Lewin, Hube *et al*. 2019). The loss of some of these genes may have been the first step toward host adaptation and pathogenicity, for example in certain fungi that cause American endemic mycoses, like *Coccidioides* spp. and *Histoplasma capsulatum* (Kohler, Hube *et al*. 2017). These species are also considered primary pathogens, meaning that they can cause disease in immunocompetent hosts. The loss of enzymes that can digest plant matter allowed these typical soil-dwelling fungi to specialize in degrading animal matter, eventually being able to infect mammalian hosts, like rodents and bats (Figure 2) (Sharpton, Stajich *et al*. 2009, Siscar-Lewin, Hube *et al*. 2019). *H. capsulatum* can infect bats and is well adapted to the environment of the phagolysosome, being able to survive within macrophages with low inflammation and eventually promoting chronic infections (Taylor, Chavez-Tapia *et al*. 1999, Horwath, Fecher *et al*. 2015). In humans, this chronic infection in immunocompetent hosts is usually asymptomatic (Kandi, Vaish *et al*. 2016). Similarly, *Coccidioides* spp. can cause mild respiratory infections, of which the majority (60%) are asymptomatic (Galgiani, Ampel *et al*. 2005), and in rare occasions it (maybe "accidentally") can reach the central nervous system (Hector, Rutherford *et al*. 2011). Furthermore, in many commensal and environmental fungi, the loss of antivirulence genes increases immune evasion and stress resistance, which specifically increases fungal fitness within the mammalian host (Siscar-Lewin, Hube *et al*. 2019). These examples support the assumption that the evolution of fungal virulence with its loss of antivirulence genes seems to promote

persistence rather than direct host damage, even in primary pathogenic fungi, unlike what is generally observed in bacterial obligate pathogens (Ehrt, Schnappinger *et al*. 2018, Demeure, Dussurget *et al*. 2019). However, in the special case of immunocompromised patients, fungal phenotypes that usually confer resistance and survival in the host can cause severe damage due to their increased susceptibility.

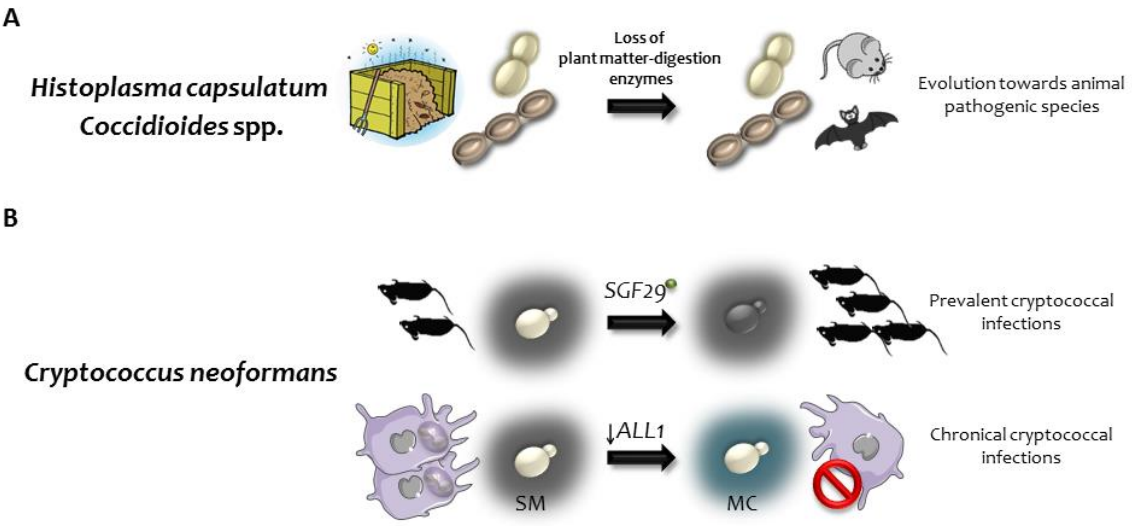

Figure 2. The loss of antivirulence genes as a hallmark of evolution towards virulence. A) The loss of genes involved in degrading decaying plant matter is possibly an important step during the evolution of certain plant saprophytic fungi to animal-associated and pathogenic fungi (Moran, Coleman *et al*. 2011). **B)** Spontaneous inactivating mutations in the gene *SGF29* lead to increased melanization, which is associated with an increase in virulence in animal models. These mutations have been found in patients with prevalent cryptococcal infections (Arras, Ormerod *et al*. 2017). The downregulation of *ALL1* gene promotes phenotypic switching in *C. neoformans* with a change in capsule polysaccharide composition, which inhibits phagocytosis and impairs cell-mediated immune responses. This has been shown to happen during chronic cryptococcal infections (Jain, Li *et al*. 2009). SM: smooth colony variant, MC: mucoid colony variant.

During infection, opportunistic fungi face stressful conditions derived from the host immune responses (e.g. phagocytosis, oxidative stress, acidic pH, enzymatic digestion, and starvation), and antifungal treatment. The presence of regulatory mechanisms to switch to more virulent phenotypes on demand would confer an adaptive advantage for opportunistic pathogenic fungi to evade or surpass the immune responses and survive antifungals, promoting persistence. The inactivation or loss-of-function mutation of antivirulence genes lead to more adaptive phenotypes during infection as it is the case of the gene *SGF29* (encoding SAGA-acetyltransferase associated factor Sgf29), in the opportunistic pathogen *C. neoformans*, whose loss of function lead to an increase in melanization and in turn better fitness during infection and hypervirulence (Arras, Ormerod *et al*. 2017). Importantly, *SGF29* loss-of-function mutations have been found in

clinical isolates from patients with prevalent infections, underlining the importance of loss of antivirulence genes in the development and outcome of an infection (Arras, Ormerod *et al*. 2017) (Figure 2).

Fungi can also adapt their physiology to changing environments by a mechanism called phenotypic switching. This mechanism is a cellular process that enables microbes to adapt to fluctuating environments undergoing rapid changes without the handicap of accumulating disadvantageous mutations (Jain, Guerrero *et al*. 2006). Importantly, phenotypic switching is a reversible mechanism. This process has been observed for pathogenic fungi, defined as spontaneous emergence of colonies with altered colony morphology as well as changes on the cellular level (Soll 1992). *C. neoformans* relies on phenotypic switching between a virulent mucoid form (MC) and a smooth colony variant (SM) during human infection to evade clearance by the immune responses and/or antifungal treatment, promoting infection persistence (reviewed by Jain, Guerrero *et al*. 2006). There are several possible molecular mechanisms for phenotypic switching in human pathogenic microbes: The downregulation of antivirulence genes like *ALL1* (Allergen 1) involved in the structure of the capsule polysaccharide has been shown to be involved in phenotypic witching from SC to MC (Jain, Li *et al*. 2009) (Figure 2); a downregulation of transcription has also been shown in *C. albican*s and *C. neoformans* (Sonneborn, Tebarth *et al*. 1999, Jain, Li *et al*. 2009) and the inactivation of genes by mutation is known for example in *Pseudomonas aeruginosa* (Martin, Schurr *et al*. 1993). Other cases of a loss which promotes phenotypic switching and higher colonization and persistence are respiratory or mitochondrial mutants, such as the numerous bacterial SCVs, but also the *petite* phenotype of *C. glabrata* (Day, 2013; Kahl *et al*. 2016). The fact that the absence of those genes' and physiological functions is triggering a more virulent phenotype classifies them as potential antivirulence genes (Siscar-Lewin, Hube *et al*. 2019).

In conclusion, it is clear that fungal pathogenicity has emerged independently and has taken multiple directions, even in closely related species, which makes it difficult to understand the biological niches and evolutionary pressures that gave rise to these pathogens. The co-evolution of host and commensals is an example of that: On the one hand it led to the emergence of more specialized commensal species, whereas on the other hand the closest relative of these commensals have evolved towards more virulent phenotypes whose virulent factors are beneficial in conditions of host immunosuppression, but become avirulence factors in conditions of immunocompetence. In addition, the loss or inactivation of antivirulence genes in some

opportunistic pathogens, either commensals and environmental fungi, provide the grounds for the emergence to animal-associated fungi and a diversity of phenotypes that eventually contribute to the rapid adaptation to fluctuating environments, like those encountered during infection and antifungal treatment. The fact that virulence factors of commensal fungi evolve to prevent exacerbate host damage in immunocompromised host (lower virulence) and immunorecognition in immunocompetent host (lower avirulence) suggests a pathogen's evolutionary trajectory towards milder virulent forms far from the virulence exhibited by a obligate pathogens, but rather persistence.

7.2 Mitochondria as an antivirulence factor

As introduced above, phenotypic switching is an important strategy that microorganisms like yeasts evolved to achieve heterogeneity. Heterogenous populations can survive better in fluctuating and stressful environments, since they will more likely include individuals that can survive and thrive under a given condition. Here applies the concept of bet hedging strategy, which occurs when some phenotypes show a decreased fitness in their typical conditions in exchange for increased fitness in stressful conditions (Holland, Reader *et al*. 2014). Besides *C. neoformans*, other important opportunistic pathogens, like *C. albicans* also undergo phenotypic switching during infection (Jain, Guerrero *et al*. 2006, Alby and Bennett 2009) allowing them to evade the immune responses and promoting persistence. In *C. albicans*, the switch between white and opaque phenotypes is modulated in response to multiple forms of cell stress, such as oxidative stress. The opaque phenotype is less efficiently phagocytosed by macrophages and does not secrete a chemoattractant, which is perceived by leukocytes and promotes inflammation, as the white phenotype does (Alby and Bennett 2009). Therefore it is thought that the transition to opaque cells could promote immune evasion and better survival to the oxidative stress generated by the immune system during infection (Alby and Bennett 2009). Whether this proposed principle is linked to the different mating potential of *C. albicans* opaque *versus* white switching phenotypes (Miller and Johnson 2002) remains to be investigated.

C. neoformans, as mentioned above, undergoes phenotypic switching during chronic infections, which promotes immune evasion and enhances virulence (Jain, Guerrero *et al*. 2006). Furthermore, by comparing the growth of two *S. cerevisiae* populations with different switching rates, it was shown that the adaptive value of the ability for rapid phenotype switching depends on the frequency of environmental changes. The fast-switching phenotype outgrew the slow switcher under rapidly environment fluctuations, and *vice versa* when fluctuations were rare. This suggests that cells may adjust their

phenotype switching rates to the frequency of environmental changes (Acar, Mettetal *et al*. 2008). This would predict a high spontaneous rate of emergence of SCVs in bacterial pathogens and *petites* in yeast upon frequent, stressful changes in the environment, such as temperature and carbon sources, if these phenotypes can be adaptive (Day 2013, Tuchscherr, Pollath *et al*. 2019).

According to the data obtained in this work, a lack of mitochondrial function may provide an advantage for *C. glabrata* during infection. Despite the fitness impairment under optimal growth conditions due to this dysfunction, here it was shown that it also confers two efficient adaptation traits: On the one hand the resistance to antifungal drugs is significantly increased, and on the other hand early survival in host phagocytes is much higher. These additional resistances should exert a strong selection in favor of the *petite* phenotypes during typical human infections. It is therefore reasonable to assume that they have clinical significance and that the *petite* phenotype is a bet-hedging strategy in which the yeast is able to cope with adverse conditions at a price of reducing overall growth (Lowery, McNally *et al*. 2017). Nevertheless, recent experimental evolution approaches have shown that *C. glabrata* virulence does not always correlate positively with its growth rate, and so the costs of resistance and lack of respiratory metabolisms of the *petites* do not necessarily interfere with a virulent phenotype (Duxbury, Bates *et al*. 2020).

While the immediate advantages of losing mitochondrial function are clear from the data, it still remains unknown which external factors and selection pressures led to *C. glabrata*'s ability to switch to the *petite* phenotype. One can speculate that the cross-resistance of *C. glabrata petite* between at least two major stresses during infection (antifungals and phagocytosis) is a very beneficial adaptation to the host in general, which may have its roots in its commensal lifestyle. In addition the ER stress resistance is an important adaptive advantage to the host environment since it is known that ER stress is a relevant general stress that pathogens regularly face during infection (Kaur, Ma *et al*. 2007, Cohen, Lobritz *et al*. 2013, Tiwari, Thakur *et al*. 2015). First, the cross-resistance phenomenon as well as the ER stress is consistently observed in different *C. glabrata* isolates, but is absent in *S. cerevisiae*, although other *petite*-defining features are identical. Secondly, *CgMIP1* shows the highest positive selection ratio among all genes in the evolutionary line leading to *C. glabrata*. It seems unlikely to be coincidence that this gene is involved in the replication and maintenance of mtDNA, the principal factor that determines the *petite* phenotype.

How the initiation of the *petite* phenotype is triggered *in vivo* remains unknown. The lack of a common specific mutation pattern in the *CgMIP1*-coding sequences of the *petite*

clinical strains makes it unlikely that a (reversible) mutation is involved in this switch of phenotype. An alternative explanation is an epigenetic regulation, which has been similarly suggested in the past to play a role in mitochondria-related azole resistant (Kaur, Castano *et al*. 2004). Interestingly, the variation in the mitochondrial signal peptide (MSP) between the *petites* and the respiratory-competent clinical strains observed here offers another hypothesis to explain a potential phenotypic switching: A protein with an altered, possibly less strong MSP may not be always directed correctly to the mitochondria, especially under stressful conditions where increased protein misfolding (ER stress) triggers a stricter sorting, and eventually the mtDNA would not be duplicated. As a consequence the number of mitochondrial polymerase molecules would be reduced and so would the mitochondrial DNA and lastly, function, triggering the *petite* phenotype.

The stability and reversibility of the *petite* phenotype constitute another important factor in its adaptive potential. While the *petite* phenotype confers an adaptive advantage during infection and antifungal treatment, in the absence of these stressors the same phenotype would strongly impair *C. glabrata* fitness even in its normal commensal environment. Due to its slow growth, it most likely would be quickly outcompeted by the surrounding microorganisms and the non-*petite* yeasts. Although mitochondrial function can in principle be recovered, our study shows that the majority of *petites* that arise *in vitro* and *in vivo* have lost their mtDNA and show an irreversible *petite* phenotype. It seems possible that this might be a result of an imperfectly working switching mechanism between *petite* and wild type. In this model, *CgMIP1* would switch between activation and inactivation depending on the external stress conditions and control mitochondrial function through synthesis of mtDNA, where at a low threshold the mitochondrial function is reduced to near zero, but some mtDNA molecules remain as templates for future DNA replication. Since this mechanism may not be precisely regulated in the still evolving pathogen yet, all mtDNA is lost in many cases and with it the ability to revert to wild type. The variations in the MSP sequences of clinical strains are compatible with this scenario, as DNA mutations are known to revert in commensal and pathogenic bacteria giving rise to phenotypic diversity (Moxon, Bayliss *et al*. 2006, Bayliss 2009, Palmer, Lipsitch *et al*. 2013). In those cases, certain genes show Simple Sequence Repeat (SSR) tracts in their sequence where the DNA replication is less efficient and change the number of repetitions causing a shift of the downstream reading frame. This results in incorrect translation, and consequently a “switching off” of the gene. These mutations are called phase-variable and are both, frequent and reversible, leading to rapid, stochastic on-off switching of gene expression and the associated phenotype. They can exist even before the onset of selective pressure “prime” the organism for rapid adaptation (Palmer,

Lipsitch *et al*. 2013). Such a mechanism has been shown for the important bacterial pathogen, *Yersinia pestis*. This pathogen has evolved from *Yersinia pseudotuberculosis* by many losses of antivirulence genes, and unlike *Y. pseudotuberculosis*, it also shows a phase-variable mutation in the *ureD* gene, which can switch from wild type sequence to mutant, and *vice versa* (Sebbane, Devalckenaere *et al*. 2001, Chouikha and Hinnebusch 2014, McNally, Thomson *et al*. 2016). The *ureD* gene that is inactivated by these spontaneous mutations encodes the urease accessory protein necessary for the function of the urease enzyme, whose activity would be toxic in the pathogen's flea vector. Once the bacterium leaves one of the host organisms, the flea, *ureD* mutation spontaneously reverts and urease function is recovered for the stages of its life cycle when it is needed (Sebbane, Devalckenaere *et al*. 2001, Chouikha and Hinnebusch 2014, McNally, Thomson *et al*. 2016). If an analogous mechanism is to be assumed for *CgMIP1*, further research is needed to elucidate whether the changes in the MPS are frequently found in clinically isolated *C. glabrata petites,* whether their frequency differs in different geographical zones, and whether these mutations lead to significant structural changes of the MPS which affect its function.

Furthermore, it has been shown that point mutations in the polymerase domain of the orthologous *MIP1* gene in *S. cerevisiae* triggered the emergence of *petites*, and this frequency increased with higher temperatures (28°C and 36°C). Specifically, the mutation E900G yielded from 6% *petites* at 28°C to 92% at 36°C (Baruffini, Ferrero *et al*. 2007). Of these *petites*, 33% and 88%, respectively, were completely devoid of mtDNA, referred to as rho^0. The remaining *petite* contained amplified mtDNA fragments that maps to various positions of the mtDNA, referred to as rho^- (Baruffini, Ferrero *et al*. 2007). These retained mtDNA fragments could rescue the wild type phenotype by homologous recombination when the strain was crossed with a respiratory-deficient mutant harboring mutated mtDNA, as observed before (Tzagoloff, Akai *et al*. 1975, Baruffini, Lodi *et al*. 2006). Interestingly, the wild type strain of *C. glabrat*a and its two most related human pathogenic associated species, *N. nivariensis and N. bracarensis* show the substitution E926D compared to the homologous and *petite* frequency-related E900 position in *S. cerevisiae*. In contrast, the related environmental species *N. delphensis*, *N. bacillisporus* and *C. castelli* share the residue at this position (E926) with *S. cerevisiae*. Moreover, we also found that some genomic DNA of the sequenced *petites* showed mtDNA fragments, similar to the *S. cerevisiae* rho^- strains, although so far we did not test whether they could serve as a template to rescue mtDNA and restore the wild type phenotype. Such rescue by crossing would therefore be possible if *C. glabrat*a mating can happen *in vivo,* which many recent publications indicate so, as this species has conserved the majority of the

genes required for mating and cell type identity (Yanez-Carrillo, Robledo-Marquez *et al*. 2014) and genomic footprints of recombination shows compelling evidence that this yeast is able to have sex (Gabaldon and Fairhead 2019), although it has never been shown *in vitro*.

These findings support the argument that *MIP1* has evolved differently, at least in the pathogenic species most closely related to *S. cerevisiae* that show a mutation in *MIP1*, which similarly what happens in *S. cerevisiae*, leads to high frequency of *petites*. In addition, some *C. glabrata* clinically isolated *petites* seem to be rho^{-} (partial loss or mutated mtDNA) rather than rho^{0} (complete loss of mtDNA), and may still retain the ability to restore mitochondrial function when crossed with respiratory-deficient mutants harboring point mutations in genes encoding respiratory proteins, similarly to *S. cerevisiae* (Tzagoloff, Akai *et al*. 1975, Baruffini, Lodi *et al*. 2006).

In conclusion, the loss of an important fungal physiological function, like mitochondrial activity, can lead to beneficial and adaptive consequences under stress that outweigh the fitness impairment by the functional loss. The mitochondrial polymerase Mip1 therefore constitutes the first example of a potentially reversible antivirulence factor in a human fungal pathogen with clinical importance.

7.3 Clinical relevance of the *petite* phenotype

In the human body, there are a number of scenarios in which the *petite* phenotype can be selected for and become a health threat: 1) In the gut and during inflammation, yeasts are faced with low oxygen levels (Anand, Gribar *et al*. 2007, Nizet and Johnson 2009, Zheng, Kelly *et al*. 2015) where the lack of respiratory growth in *petites* is not disadvantageous anymore, since the wild type cannot use the mitochondrial respiratory chain. In this scenario wild type and *petite* would have comparable metabolic capacities, but the *petites* still would have the advantage of a higher stress resistance. 2) A high number of fungal infections happen in patients under immunosuppression due to treatment with chemotherapeutics (Badiee and Hashemizadeh 2014). It has been shown that *S. cerevisiae* turns *petite* in presence of chemotherapeutics used in the clinics, since many of them negatively affect mitochondria function (Buschini, Poli *et al*. 2003, Gorini, De Angelis *et al*. 2018). A similar effect seems likely for *C. glabrata*, but has not yet been established. 3) Upon infection *C. glabrata* will encounter phagocytes like macrophages, and the presence of ROS in the phagosome can impair mitochondrial function, thereby triggering *petite* phenotypes, which will endure early stages after phagocytosis better than the wild type. While the *petite* phenotype is disadvantageous when staying intracellularly for a long time, as shown at least *in vitro* by the data presented here, *petite*

cells might escape the immune cells before this disadvantage becomes visible. Alternatively, a sub-population of *petites* might benefit from the replication efficiency of an otherwise wild type population which can burst the macrophage within the first days (Seider, Brunke *et al*. 2011). Finally, *petite* cells could make use of a non-lytic escape route as it has been reported for other yeasts, like *C. albicans* and *C. neoformans* (Seoane and May 2020), although this has not been observed yet for *C. glabrata*. 4) In patients, treatment with azole antifungals will have an effect on *C. glabrata* outside the phagocytes after its escape or before phagocytosis, and this selection would likely be one of the major sources of *petite* phenotypes in the clinical setting. The use of triazoles, especially fluconazole, constitutes one of the main treatments for candidiasis (Pappas, Kauffman *et al*. 2016). Since *C. glabrata* is intrinsically resistant to such drugs, the recommendation for *C. glabrata* infections is to administer very high doses, approximately 400 to 800 mg of fluconazole per day in adults, which are around ten to twenty times the MIC_{50} (in mg ml^{-1}) reported for this fungus (Arendrup 2017). Of special clinical importance is the fact that *petites* may be able to reverse when the azole treatment ceases, and regain its general fitness. Wild type *C. glabrata* could then thrive in those scenarios where oxidative phosphorylation is advantageous and becoming *petite* is non-adaptive (*i.e.* fast replication within phagosomes with alternative carbon sources and direct competition with bacteria and other fungi) (Figure 3).

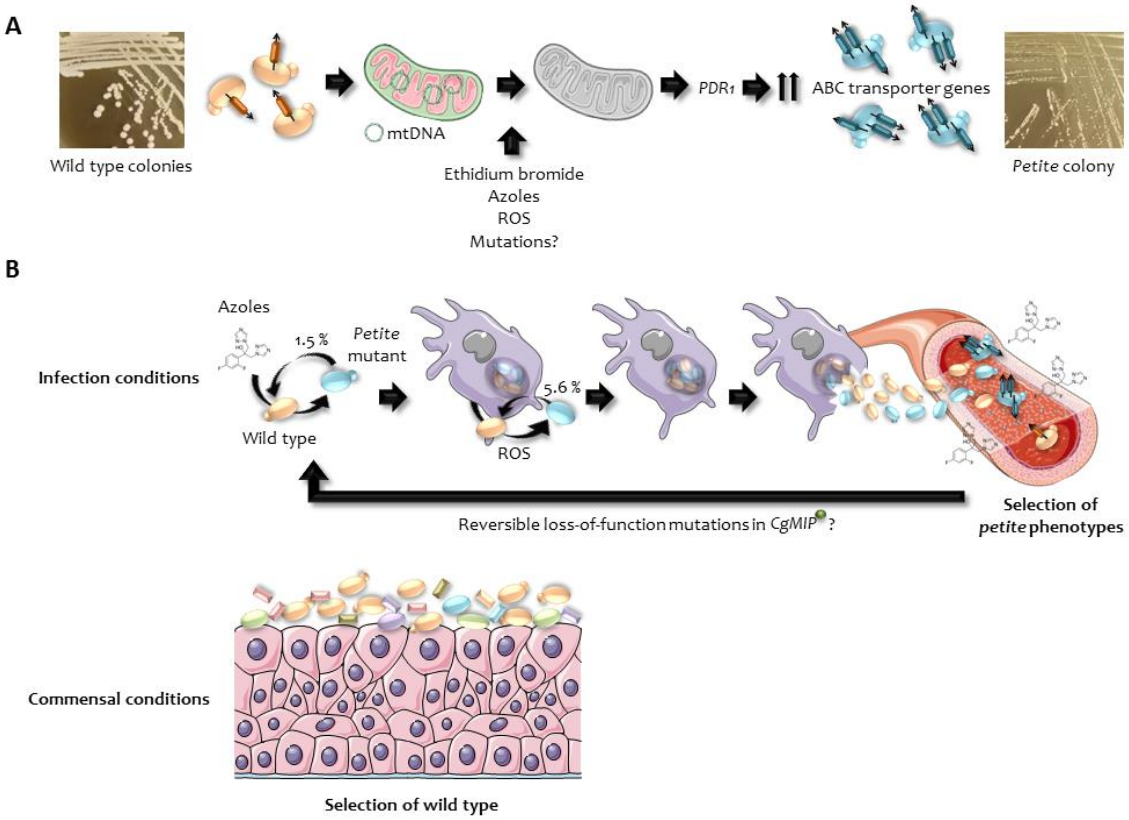

Figure 3. *C. glabrata* is a *petite*-positive yeast whose loss of mitochondrial function can become advantageous under infection conditions. **A)** Loss of mitochondrial function in *petite*-positive yeasts gives rise to small colonies with increased expression of efflux pumps-related genes, in *C. glabrata* mostly *via* upregulation of the transcription factor gene *PDR1*. **B)** Model that represents the adaptability and selection of the *petite* phenotype in *C. glabrata* during infection (phagocytosis and azole antifungal treatment) in contrast to the selection of the wild type during commensal conditions. Percentages represent the reversibility of *petite* phenotype to wild type after azole exposure and phagocytosis, respectively. Escaping *petites* can be under positive selection in the body due to continuing azole and phagocytic actions. Under commensal condition, where competition with other microbiota favors yeasts with high replication rates, the wild type is better adapted and outcompetes naturally occuring *petites*.

Nonetheless, the current clinical situation seems not to agree with the potential threat that the *petite* phenotype represents, since very few *petite* have been recovered from patients undergoing candidiasis (Bouchara, Zouhair *et al*. 2000, Posteraro, Tumbarello *et al*. 2006) and the ones analyzed in this study were only found when isolation protocols were modified in a diagnostic unit on request. Therefore, a likely explanation for this could be the inefficiency of conventional diagnostic methods in identifying *petites*, since they require cultivation of the pathogen (Kozel and Wickes 2014). The time of incubation is critical to identify *petites*: A minimum of 48 h is necessary to obtain visible colonies from *petites*, which is too long in situations where an early treatment decision is crucial. For further identification of potential *petite* colonies culture in non-fermentable carbon sources, growth on TTC agar or mtDNA detection by RT-qPCR could be useful methods, but once again they require long times. In addition, the use of chromogenic media (CHROMagar Candida, France) (Nadeem, Hakim *et al*. 2010) can also lead to misdiagnosis, as the color of *petite* colonies differs from typical *C. glabrata* and resembles *S. cerevisiae* (data not shown). If a diagnostic step could be introduced to discern *petites*, targeted treatment would become possible: One possibility would be the selection of the treatment based on first plate data (i.e. presence of *C. glabrata*), while the plates are kept incubating to assess whether *petites* appear. Then these can be analyzed in order to take further decision on the patient treatment. New diagnosis tools can be applied for these analysis that use genome sequencing and proteomics (high-throughput -omics technologies) to detect specific patterns down to the species level and potentially phenotypic diversity. Such methods would likely be able to identify *petite* mutants based on their lack of mtDNA and metabolic differences (Consortium and Gabaldon 2019).

Further research is therefore necessary to estimate the incidence and prevalence of *petite* phenotypes in the clinics. Of special importance would be to find common genetic or phenotypic patterns that can serve as diagnostic biomarkers. In addition, if the

conclusions of this work are applicable to the clinical situation, the use of triazoles, antibiotics or chemotherapeutics could lead to a possible selection of the more chemo- and immune-resistant *petite* phenotype. In this case, such a treatment should be accompanied by surveillance for an appearance of these mutants to specifically prevent and/or treat *C. glabrata petite* infections.

7.4 Further host adaptations and pathogenicity traits of *C. glabrata*

In its non-*petite* form, the capacities of *C. glabrata* to not only withstand the harsh conditions in phagosomes, but also to replicate within macrophages, has been recognized as one of its main pathogenic traits (Kasper, Seider *et al*. 2015). Part of the research in this thesis has therefore focused on a better understanding of the processes that allow this fungus to survive within macrophages without resorting to a phenotypic change. One of these processes is the trehalose metabolism. It is known that trehalose buildup contributes to stress resistance in several microorganisms, such as fungi, plants, and small invertebrates (Elbein, Pan *et al*. 2003), but also the catabolism of trehalose is used as an energy source under hunger conditions (Eleutherio, Panek *et al*. 2015) – two key issues which are encountered during phagocytosis by pathogens (Sprenger, Kasper *et al*. 2018). Three trehalases have been identified in *C. glabrata* that are responsible for the degradation of trehalose, presumably acting in different life stages and cell compartments. The deletion of any of the three or of all in combination is detrimental to the survival of *C. glabrata* after phagocytosis, but interestingly, this was only detectable after four days of co-incubation (Van Ende, Timmermans *et al*. 2021). This fact is in agreement with a potential role in carbon storage mobilization during long-term starvation. As expected, the triple mutant was significantly impaired in gut colonization in animal experiments, meaning these enzymes are relevant not only in the phagosome, but also to withstand the stressful condition (*i.e.* lack of nutrients) in the gut (Van Ende, Timmermans *et al*. 2021). These results agree with a recent publication that shows a decrease in virulence of the *ATH1* deletion mutant in *C. glabrata* (Lopes, Munoz *et al*. 2020) as well as in other fungi, like *C. albicans* and *C. parapsilosis* (Pedreno, Gonzalez-Parraga *et al*. 2007, Sanchez-Fresneda, Guirao-Abad *et al*. 2015). However, in contrast to what we observed in *C. glabrata*, in these fungi the tolerance to stress was not affected or was even higher due to the protective effect of the undigested trehalose on cellular macromolecules (Pedreno, Gonzalez-Parraga *et al*. 2007, Sanchez-Fresneda, Martinez-Esparza *et al*. 2014, Sanchez-Fresneda, Guirao-Abad *et al*. 2015). Therefore in *C. glabrata* accumulation of trehalose may play a different role, if any, in stress resistance compared to these other fungal pathogens.

The decrease in virulence of trehalase mutants of *C. albicans* and *C. parapsilosis* has been shown to be connected to defects in hyphae and pseudohyphae formation (Pedreno, Gonzalez-Parraga *et al*. 2007, Sanchez-Fresneda, Guirao-Abad *et al*. 2015). Since *C. glabrata* is not a dimorphic fungus, there must be a different explanation for this phenomenon. In this fungus instead, a lack of trehalose mobilization could explain why the deletion mutants survive phagocytosis at early time points, but are cleared at a higher rate than the wild type after several days: most likely, the phagocytosed yeasts are starving for carbon within the phagosome, but only the wild type can obtain it from storage by the degradation of trehalose.

Furthermore, trehalose is not synthesized by humans (Van Dijck, De Rop *et al*. 2002, Elbein, Pan *et al*. 2003), which raises the question about the use of trehalases by a (generally) commensal yeast like *C. glabrata*. It has been proposed that during commensal colonization this yeast may benefit from trehalose released by dead surrounding microorganisms (Lopes, Munoz *et al*. 2020), or otherwise constitute remnants of some other niche (*e.g.* environment) not associated with humans, in agreement with former speculation (Gabaldon and Fairhead 2019). On the other hand, the lack of trehalose synthesis in humans opens the possibility to use these pathways as new targets to treat fungal infections. Trehalose synthesis enzymes have been considered as a promising antifungal target before, since their deletion negatively affects the progression of fungal infections (Alvarez-Peral, Zaragoza *et al*. 2002, Van Dijck, De Rop *et al*. 2002, Perfect, Tenor *et al*. 2017). In addition, this work has now shown that trehalases as degrading enzymes would also constitute a valuable antifungal target, since yeasts lacking these enzymes show reduced virulence potential.

Finally, this thesis also provided data on the influence of acetate uptake on the survival of *C. glabrata* during long-term phagosomal residence. Despite the fact that this fungus seems to rely on alternative carbon sources to grow and replicate in glucose-poor niches like the phagosome (Kaur, Ma *et al*. 2007, Fukuda, Tsai *et al*. 2013, Chew, Ho *et al*. 2019, Chew, Brown *et al*. 2021), the lack of all putative acetate transporters (Ato) and channels (Fps) increased survival after seven days of phagocytosis. This leads to a new hypothesis about the metabolism of *C. glabrata* during long-term phagosomal residence: On the one hand, it is known that growth of *C. albicans and C. glabrata* in alternative carbon sources triggers cell wall changes and different stress-resistance phenotypes (Brown, Brown *et al*. 2014, Chew, Ho *et al*. 2019, Williams and Lorenz 2020), which influences recognition and killing by macrophages (Williams and Lorenz 2020). Thus, according to the results presented here *C. glabrata* would preferably use other alternative

carbon source to prevent a higher uptake and killing by macrophages when using acetate. This also agreed with previous observations that show that growth of wild type *C. glabrata* in acetate renders the fungi more susceptible to be taken up and killed by phagocytes (Mota, Alves *et al*. 2015), likely due to the higher mannan exposure and lower oxidative-stress resistance that has been reported for these conditions in another study (Chew, Ho *et al*. 2019). On the other hand, two of the Ato transporters were shown to be upregulated during the long-term residence of the yeast within macrophages, leading to the idea that acetate is nevertheless used under these conditions, and that the upregulation of other carboxylic acid transporters may be complementing the lack of acetate transporters and channels. This would allow a better uptake of carbon, supporting survival and growth. Further experiments are necessary to assess these hypotheses: 1) The expression of other transporters of alternative carbon sources (other carboxylic acids, fatty acids, amino acids), in the wild type and acetate-transporter mutants during a long-term phagosomal residence experiment; 2) long-term growth of the wild type in different carbon sources and a subsequent phenotypic screening on stress condition and macrophage interaction, similar to what has been previously done for *C. albicans* (Williams and Lorenz 2020); and 3) deletion and characterization of gene candidates in a long-term macrophage experiment.

Altogether, these experiments show the specific strength of the long-term macrophage co-incubation model that was only recently developed (Daniel Fischer, unpublished data, Manuscript IV and Manuscript V). The impact of certain metabolic processes only becomes visible after longer time periods within the phagosome, but are most likely highly relevant for *C. glabrata*'s pathobiology. A typical short-term phagocytosis experiment would not have been able to detect the role of the trehalose and acetate metabolism in the survival and persistence of *C. glabrata* during phagocytosis.

7.5 Conclusion

In the present work new concepts to broaden our understanding on fungal pathogenesis and its evolution have been developed. These will aid further research to uncover key factors involved in fungal pathogenicity and the host’s protective responses, a crucial step to develop successful strategies to prevent, diagnose, and treat fungal infections. Specifically, this works shows that the potential to lose and gain gene functions is an important driver for pathogenicity *in vitro* and likely in the clinic. As an example for this, the *petite* phenotype has been identified as a pathogenic strategy of the opportunistic fungus *C. glabrata,* which despite its fitness cost due to loss of mitochondrial function, shows adaptability to conditions of infection and antifungal treatment. Importantly, in this

work it was possible to observe the switch from wild type to *petite* mutants only through *in vitro* long-term exposure to human macrophages, which presumably emulates the *in vivo* situation better than the conventional short-term protocols (Seider, Gerwien *et al*. 2014). In agreement with that, in 2010 Jacoben *et al*., concluded that *C. glabrata* relies on yet unknown immune evasion strategies that enable the fungi to survive, disseminate, and persist within mammalian hosts, and even when infection was performed with amino acid auxotrophic mutants they disseminated and persisted in immunocompetent mice similarly to the wild type (Jacobsen, Brunke *et al*. 2010). Therefore this method could do and has allowed us to show that *C. glabrata* uses further specific adaptations for long-term macrophage survival, such as the use of different available carbon sources (storage trehalose from within the fungus and acetate from the phagosome), which could be at work during *C. glabrata'*s survival, dissemination and persistence *in vivo*. Clearly, having a look beyond what the reference strain is doing in the short-term under standard laboratory conditions is a worthwhile approach to decipher *C. glabrata*'s virulence strategies, and therefore we need to shift the focus on finding more "life-like" accessible experimental set-ups, and this new model is an excellent compromise between *in vivo* and simpler *in vitro*. Moreover, these adaptations seem very distinct from the closely related yeast *S. cerevisiae*, indicating that the same genes or functions have been adapted in *C. glabrata* to the specific requirements in the host niche. Altogether, the results presented here therefore highlight the very specific physiological pathways that help to make this fungus a successful pathogen of humans.

8. References

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9. Appendix

9.1 Abbreviations

ABC	ATP binding cassette
ATP	adenosine triphosphate
DAMPs	Damage-Associated Molecular Patterns
e.g.	latin: *exempli gratia*, for example
GOF	gain-of-function
HR	hyper-sensitive response
i.e.	latin: *id est*, that is to say
MC	mucoid colony
MIC	minimum inhibitory concentration
MSP	mitochondrial signal peptide
mtDNA	mitochondrial DNA
NCAC	non-*Candida albicans Candida*
NETs	Neutrophil Extracellular Traps
PAMPs	Pathogen-Associated Molecular Patters
SC	smooth colony
SCV	Small Colony Variants
SSR	Simple Sequence Repeat
ROS	reactive oxygen species
VVC	vulvovaginal candidiasis

9.2 *Curriculum vitae*

9.2.1 Personal information

Name: Sofía Siscar Lewin

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9.2.2 Education and training

JANUARY 2016 - SOMETIME 2021

Doctor of Philosophy (PhD). Department of Microbial Pathogenicity Mechanisms. Hans Knöll Institute Jena (HKI) and The Excellence Graduate School Jena School for Microbial Communication (JSMC). Friedrich Schiller University (FSU) of Jena. Germany. Thesis title: Host adaptation, avirulence and antivirulence genes of *Candida glabrata.*

OCTOBER 2014 - SEPTEMBER 2015

Research Master Degree in Molecular Biology, Cell Biology and Genetics. Department of Biochemistry and Molecular Biology. University of Valencia, Spain. Thesis title: Chromatin structure and epigenetic modifications at the transcription start site and alternative splicing of the gene *ZNF518B* depending on the mutation G13D of the gene *KRAS* in colorectal cancer.

SEPTEMBER 2008 - SEPTEMBER 2014

Bachelor in Biological Sciences (Licenciatura). University of Alcalá, Spain.

9.2.3 Work experience

SEPTEMBER 2015 - DECEMBER 2015

Research Grant (Spanish Ministry of Education, Culture and Sport). Department of Biochemistry and Molecular Biology. University of Valencia, Spain. Project Title: Role of the gene *ZNF518B* in colorectal cancer.

SEPTEMBER 2013 - JULY 2014

Erasmus Internship. Department of Microbiology and Biotechnology. Eberhard Karls University of Tübingen, Germany. Project Title: Characterization of the cell wall hydrolytic enzyme YocH in *Bacillus subtilis*.

SEPTEMBER 2012 - JULY 2013

Research Grant (Spanish Ministry of Education, Culture and Sport). Department of Microbiology and Parasitology. University of Alcalá, Spain. Project Title: Bio-degradation of quinolones antibiotics using *Streptomyces ipomoea* and its laccase enzyme as bioremediatior.

9.2.4 Teaching

OCTOBER 2018 - JANUARY 2020

Supervision of Master thesis project by Franziska Pauline Pieper, FSU Jena, Germany. Thesis title: The role of the putative vacuole transporter Ctr2 of *Candida glabrata* under copper stress and during macrophage interaction.

SEPTEMBER 2019 AND SEPTEMBER 2017

Supervision of Master in Biochemistry practical course, Department of Microbial Pathogenicity Mechanisms, HKI and FSU Jena, Germany.

SEPTEMBER 2014

Master in Microbiology practical course. Department of Microbiology and Biotechnology, Eberhard Karls University of Tübingen, Germany.

9.2.5 Outreach and science communication activities

JANUARY 2019

“Dance your PhD” Competition. Title: *Candida* infections: a disbalance in the interaction with the human host and its microbiota. Jena, Germany.

NOVEMBER 2019 AND 2017

Long Night of Science, Jena, Germany.

JULY 2018

8th Science Slam Competition of Graduate Academy. Title: The black sheep of the brewer's yeast dynasty. Jena, Germany.

MARCH 2017

Co-Organizer of the 6th International Conference in Microbial Communication for Young Scientists (MiCom). Jena, Germany.

9.2.6 Conferences

POSTERS

2020 Statusworkshop Eukaryotic Pathogens. Innsbruck, Austria. Mitochondrial function as an antivirulence factor in *Candida glabrata*. 8th FEBS Advanced Lecture Course on Human Fungal Pathogens - Molecular Mechanisms of Host-Pathogen Interactions and Virulence 2019, La Colle sur Loup, France.

2018 The loss of the antivirulence gene *CgMIP1* leads to stress resistance in *Candida*

glabrata. 6th Central European Summer Course (CESC) on Mycology and 3rd Rising Stars in Mycology Workshop, Szeged, Hungary. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

20th Congress of the International Society for Human and Animal Mycology (ISHAM), Amsterdam, The Netherlands. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

7th International Conference in Microbial Communication for Young Scientists (MiCom), Jena, Germany. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

2017 7th Congress of the European Microbiologists (FEMS), Valencia, Spain. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

Microbiology and Infection - 5th Joint Conference of the DGHM & VAAM, Würzburg, Germany. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

2015 18th European Cancer Congress, Vienna, Austria. Nucleosome occupancy and epigenetic modifications in the alternative splicing site of the gene *ZNF518B* in colorectal cancer.

38th Congress of The Spanish Society of Biochemistry and Molecular Biology, Valencia, Spain. Nucleosome occupancy and epigenetic modifications in the promoter and alternative splicing sites of the gene *ZNF518B* in colorectal cancer.

2013 24th Congress of The Spanish Society of Microbiology, Barcelona, Spain. Fluoroquinolones degradation in alkaline conditions by LMS system of *Streptomyces ipomoea* CECT 3341. Process validation *in vivo*.

TALKS

2020 Statusworkshop Eukaryotic Pathogens, Innsbruck, Austria. Mitochondrial function as an antivirulence factor in *Candida glabrata.*

2019 8th FEBS Advanced Lecture Course on Human Fungal Pathogens - Molecular Mechanisms of Host-Pathogen Interactions and Virulence, La Colle sur Loup, France. The loss of the antivirulence gene *CgMIP1* leads to stress resistance in *Candida glabrata*.

2018 6th Central European Summer Course (CESC) on Mycology and 3rd Rising Stars in Mycology Workshop, Szeged, Hungary. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

6th International Conference in Microbial Communication for Young Scientists (MiCom), Jena, Germany. Studying the role of *CgMIP1* in the evolution of *Candida glabrata* during adaption to the human host.

2013 24th Congress of The Spanish Society of Microbiology 2013, Barcelona, Spain. Fluoroquinolones degradation in alkaline conditions by LMS system of *Streptomyces ipomoea* CECT 3341. Process validation *in vivo*.

9.2.7 Awards

2018 Best Oral Talk Prize at the 6th Central European Summer Course (CESC) on Mycology and 3rd Rising Stars in Mycology Workshop, Szeged, Hungary.

Federation of European Microbiological Societies (FEMS)/CESC Travel Grant.

International Society for Human and Animal Mycology (ISHAM) Travel Grant.

Jena School for Microbial Communication (JSMC) Travel Grant.

2015 Outstanding Master Student Research Grant. Spanish Ministry of Education, Culture and Sports, University of Valencia, Spain.

2013 Selected participant at the 17th Introduction to research in Microbiology Course and the 24th Congress of Spanish Society of Microbiology, Barcelona, Spain.

2012 Outstanding Bachelor Student Research Grant. Spanish Ministry of Education, Culture and Sports, University of Alcalá, Spain.

2008 Honours in Highschool, IES María Zambrano., Segovia, Spain.

9.2.8 Languages

NATIVE LANGUAGE: Spanish

OTHER LANGUAGE(S):

English: First Certificate of English (FCE). Cambridge English (2015)

German: B2 Goethe Certificate. Goethe Institute (2020)

9.2.9 Peer reviewed publications

2021 **Siscar-Lewin, S**., Gabaldón, T., Hube, B., & T., Brunke, S., Hube, B. (2021).

Transient mitochondria dysfunction confers fungal cross-resistance between macrophages and fluconazole. *mBio*. DOI: 10.1128/mBio.01128-21

Pekmezovic M, Hovhannisyan H, Gresnigt MS, Iracane E, Oliveira-Pacheco J, **Siscar-Lewin S**., *et al* (2021). *Candida* pathogens induce protective mitochondria-associated type I interferon signalling and a damage-driven response in vaginal epithelial cells. *Nature Microbiology*. DOI: 10.1038/s41564-021-00875-2

Van Ende, M., Timmermans, B., Vanreppelen, G., **Siscar-Lewin, S**., *et al*. (2021). The involvement of the *Candida glabrata* trehalase enzymes in stress resistance and gut colonization. *Virulence*, 12:1, 329-345. DOI: 10.1080/21505594.2020.1868825

Alves, R., **Siscar-Lewin, S**., *et al*. Acetate assimilation is an integral part of *Candida glabrata* persistence within the human host (In preparation)

2019 **Siscar-Lewin, S**., Hube, B., & Brunke, S. (2019). Antivirulence and avirulence genes in human pathogenic fungi. *Virulence*, 10(1), 935-947. DOI: 10.1080/21505594.2019.1688753

Gimeno-Valiente, F., Riffo-Campos, Á. L., Vallet-Sánchez, A., **Siscar-Lewin, S**., *et al*. (2019). *ZNF518B* gene up-regulation promotes dissemination of tumor cells and is governed by epigenetic mechanisms in colorectal cancer. *Scientific Reports*. 9(1), 9339. DOI: 10.1038/s41598-019-45411-9

9.3 Candidate's contribution to original publications

Manuscript I – Siscar-Lewin *et al* (2019), *Virulence*

Contribution of the doctoral candidate to Figures that reproduce experimental data:

Figure 1-2	☒	100%

Manuscript II - Pekmezovic, Hovhannisyan *et al* (2021), *Nat Microbiol*

Contribution of the doctoral candidate to Figures that reproduce experimental data:

Figure 1	☒	20% (Generation times of *Candida* species (B))
Extended data Figure 2, 3, 6, Figure 1-4, 6-7, Supplementary files 1-10, Supplementary tables 1-2	☐	0% (The data shown in this Figure are based exclusively on the work of other coauthors)
Figure 4	☒	15% (Mitochondrial membrane potential (E))
Figure 5	☒	50% (mtDNA quantification (A) and ISG expression (B))
Extended data Figure 5	☒	25% (Mitochondrial membrane potential of primary cells (C))
Extended data Figure 8	☒	100% (The data shown in this Figure come entirely from experimental work carried out by the candidate)

Manuscript III – Siscar-Lewin *et al* (2021), *mBio*

Contribution of the doctoral candidate to Figures that reproduce experimental data:

Figure 1-6	☒	100%
Figure 7	☒	25% (DNA isolation and Figure)
Supplementary files Figures 1-3	☒	100%

Manuscript IV –Van Ende, Timmermans *et al* (2021), *Virulence*

Contribution of the doctoral candidate to Figures that reproduce experimental data:

Figure 1-5 and 7	☐	0% (The data shown in this Figure are based exclusively on the work of other coauthors)
Figure 6	☒	75% (Experiment design, work and analysis)
Supplementary files	☐	0% (The data shown in this Figure are based

Figures 3-9		exclusively on the work of other coauthors)

Manuscript V – Alves *et al*, *In preparation*

Contribution of the doctoral candidate to Figures that reproduce experimental data:

Figure 1-5	☐	0% (The data shown in this Figure are based exclusively on the work of other coauthors)
Figure 6	☒	80% (Experiment design, work and analysis)

9.4 Selbstständigkeiterklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbst verfasst habe und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Mir ist die geltende Promotionsordnung der Biologisch-Pharmazeutischen-Fakultät der Friedrich-Schiller-Universität Jena bekannt. Personen, die mich bei den Experimenten, der Datenanalyse und der Verfassung der Manuskripte unterstützt haben, sind als Ko-Autoren auf den entsprechenden Manuskripten verzeichnet. Personen die mich bei der Verfassung der Dissertation unterstützt haben, sind in der Danksagung der Dissertation vermerkt. Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen. Es haben Dritte weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die vorliegende Arbeit wurde in gleicher oder ähnlicher Form noch bei keiner anderen Hochschule als Dissertation eingereicht und auch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung verwendet.

Jena, den 10.05.2021

_____

Sofía Siscar Lewin

9.5 Acknowledgments

First of all, I would like to express my deepest gratitude to Prof. Bernhard Hube for trusting and giving me the opportunity to conduct this project at the Department of Microbial Pathogenicity Mechanisms in the Hans Knöll Institute. I especially want to thank for his patience, his professional lead and his warming encouragement.

Secondly, I thank Dr. Sascha Brunke for his supervision, his corrections and smart advice, his patience and his ability to calm me down during my panicking phases.

I also thank the Jena School for Microbial Communication for funding my PhD and being a fantastic support and teaching platform, through which I have gained knowledge on relevant topics beyond my PhD.

I am greatly thankful to have shared a wonderful work environment with my colleagues of the Department of Microbial Pathogenicity Mechanisms, Microbial Immunology and Adaptive Pathogenicity Strategies. Thanks for the great team work, help, positivisms, enthusiasm and fun time. I would like to specially thank Marina Pekmezovic, Osama Elshafee, Franziska Pieper, Ann-Kristin Kaune, Theresa Rothe, Marisa Valentine, Raquel Alonso (Leída, Faraona), Jakob Sprague, Toni Förster, Raghav Vij, Stephanie Wisgott, Daniela Schulz, Verena Trümper, Franzi Gerwien, Annika König (Annikita), Stefanie Allert, Antonia Last, Marcel Sprenger, Mark Gresnigt, Sophie Austermeier, Sophia Hitzler, Rita Müller, Bettina Böttcher, Philipp Kämmer, Volha Skrahina, and last but not least the HKI toilets for being a safe place when I needed escape.

I also thank the collaboration partners with whom I have learnt and enjoyed working with: Prof. Toni Gabaldón (Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain), Mieke Van Ende (Laboratory of Molecular Cell Biology, Department of Biology, Institute of Botany and Microbiology, Leuven, KU Leuven, Belgium), Dr. Rosana Alves (Department of Biology, Centre of Molecular and Environmental Biology, School of Sciences, University of Minho, Braga, Portugal) and Alexander M. Aldejohann (Institute for Hygiene and Microbiology, Julius-Maximilians-University, Josef-Schneider-Straße 2, 97080 Würzburg, Germany).

This doctoral thesis has also been a very influential journey to me as a person; I feel I did not only accomplish a higher educational degree in science but also in life. This period has brought me closer to my true self. As a consequence I have learnt to perceive and deal with my insecurities, fears, weaknesses and frustrating expectations; but I have also

realized about my undeniable self-awareness and world perception, courage, determination and perseverance. All this has been indisputably possible thanks to the wisdom of Borja Vilaseca, Marina García, Julia Gutiérrez, Marina Pekmezovic and Aleksa Stanisic. In this part I especially thank Marina Pekmezovic to be my lab partner and inseparable friend that has always helped, encouraged and comforted me in the hardest moments of the PhD life, and enjoyed to the fullest (also understood as quality of life) in the nice days. Together with Aleksa Stanisic, we have turned the bitterest moments into laugh and joy during our lunch breaks. Thanks Aleksa for making me laugh so much and helping me “chemically” when my mind needed an urgent reset. I cannot be more grateful for all the episodes (LZDLS) I have lived in Jena along with my PhD, it has definitely been a wonderful experience.