Fungal biotin homeostasis is essential for immune evasion after macrophage phagocytosis and virulence

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Abstract
Biotin is an important cofactor for multiple enzymes in central metabolic processes. While many bacteria and most fungi are able to synthesise biotin de novo, Candida spp. are auxotrophic for this vitamin and thus require efficient uptake systems to facilitate biotin acquisition during infection. Here we show that Candida glabrata and Candida albicans use a largely conserved system for biotin uptake and regulation, consisting of the high-affinity biotin transporter Vht1 and the transcription factor Vhr1. Both species induce expression of biotin-metabolic genes upon in vitro biotin depletion and following phagocytosis by macrophages, indicating low biotin levels in the Candida-containing phagosome. In line with this, we observed reduced intracellular proliferation of both Candida cells pre-starved of biotin and deletion mutants lacking VHR1 or VHT1 genes. VHT1 was essential for the full virulence of C. albicans during systemic mouse infections, and the lack of VHT1 led to reduced fungal burden in C. glabrata-infected brains and C. albicans-infected brains and kidneys. Together, our data suggest a critical role of Vht1-mediated biotin acquisition for C. glabrata and C. albicans during intracellular growth in macrophages and systemic infections.

KEYWORDS
biotin, Candida albicans, Candida glabrata, macrophage, metabolism, virulence

1 | INTRODUCTION

The ability to acquire macronutrients, such as carbon and nitrogen sources, and micronutrients, such as trace metals and vitamins, is of vital importance for all organisms. Vitamins, although required in very small quantities, are involved in numerous cellular processes (Combs & McClung, 2017; Dakshinamurti, 2005). During microbial infections, vitamin availability becomes important at the host-pathogen interface. On one hand, the host vitamin status is important for immune functions, on the other hand, pathogens need to synthesize or acquire vitamins in order to proliferate in the host (Combs & McClung, 2017; Mikkelsen, Stojanovska, Prakash, & Apostolopoulos, 2017; Stephensen, 2001). In this study, we investigate the requirement of biotin (vitamin H or vitamin B7) for human pathogenic Candida species. This vitamin is known to be crucial for key metabolic pathways, like gluconeogenesis and amino acid and fatty acid metabolism, which require the activity of
biotin-dependent carboxylases (Knowles, 1989; Tong, 2013; Zempleni, Wijeratne, & Hassan, 2009).

While mammals need to take up biotin as part of their diet (Zempleni et al., 2009), many bacterial and fungal pathogens are able to synthesise biotin de novo (Garfoot, Zemska, & Rappleye, 2014; Magliano, Filippi, Sanglard, & Poirier, 2011; Magnusdottir, Ravcheev, de Crecy-Lagard, & Thiele, 2015; Napier et al., 2012; Salaemae, Booker, & Polyak, 2016). In contrast, yeasts belonging to the Candida genus, including opportunistic pathogens (Pfifer & Diekema, 2007), are biotin auxotrophs (Littman & Miwatani, 1963; McVeigh & Bell, 1951). Medically important Candida spp. colonise the oral cavity, urogenital, or gastrointestinal tract as harmless commensals (Huffnagle & Noverr, 2013), but can also cause disease ranging from superficial mucosal to systemic infections with dissemination throughout the host to allow normal growth and virulence of fungal pathogens (Meir & Osherov, 2018). These findings, however, also implicate that fungal pathogens, especially Candida species, must possess efficient uptake systems to acquire biotin during infection. This may be especially challenging in nutrient-poor host compartments, like the macrophage phagosome. Macrophages are important effectors of the innate immune system, which can recognise, ingest, and kill microbes including Candida species (Erwig & Gow, 2016; Miramón, Kasper, & Hube, 2013). The compartmentalisation in phagosomes is assumed to be a major mechanism to control microbial growth by increasing the efficiency of antimicrobial activities, but also by restricting nutrients (Haas, 2007; Sprenger, Kasper, Hensel, & Hube, 2017). Indeed, transcriptional profiling of fungal responses to macrophage phagocytosis suggests that C. albicans and C. glabrata experience nutrient limitation inside the phagosome (Chew et al., 2019; Childers et al., 2016; Kaur, Ma, & Cormack, 2007; Lorenz, Bender, & Fink, 2004; Roetzer, Gratzi, Kovari, & Schüller, 2010). Both C. albicans and C. glabrata, however, can adapt to macrophage antimicrobial activities and phagosomal nutrient conditions, proliferate intracellularly, and escape from these phagocytes (Miramón et al., 2013). Survival strategies, although sharing basic concepts, differ between the two species. Phagocytosed C. albicans yeast cells rapidly start to form hyphae, damage macrophage membranes, and escape after host cell death and via hyphal outgrowth (Kasper et al., 2018; McKenzie et al., 2010; Vylkova & Lorenz, 2014; Westman, Moran, Mogavero, Hube, & Grinstein, 2018). In contrast, C. glabrata replicates as yeast cells, is able to persist intracellularly for several days without causing damage, and finally escapes from macrophage by unknown mechanisms (Kaur et al., 2007; Seider et al., 2011).

In this study, we investigated the relevance of biotin acquisition of C. albicans and C. glabrata for growth, during interaction with macrophages, and systemic infection.

2 | RESULTS

2.1 | Pathogenic Candida species require biotin or biotin precursors for growth

While many bacterial and fungal species are able to synthesise the essential vitamin biotin de novo, most multicellular eukaryotes (excluding plants) and many yeasts are biotin auxotrophic (Roje, 2007). To find out how different medically important Candida species cope with biotin limitation, we analysed growth of C. albicans, C. glabrata, C. dubliniensis, C. parapsilosis, C. tropicalis and C. auris in minimal medium lacking biotin or containing different biotin sources. As expected, all tested species grew well in the presence of biotin, but showed drastically reduced growth rates in the absence of biotin (Figures 1a,b and Figure S1). Growth defects in biotin-depleted media were more pronounced when C. albicans and C. glabrata were pre-starved in biotin-free medium prior to the experiment, suggesting that both yeasts can use biotin from intracellular stores (Figure 1b). In host niches like the gut, which are colonised by multiple microbes of the microbiota, Candida cells may have access to biotin biosynthesis intermediates such as 7-keto-8-aminopelargonic acid (KAPA) or desribiotin (DTB) produced by these other microbes (Hill, 1997). While C. albicans and C. tropicalis grew in the presence of either KAPA or DTB, C. dubliniensis and C. auris only grew in the presence of DTB, and C. glabrata and C. parapsilosis were unable to effectively utilise these biotin intermediates (Figures 1a,b and S1a).

Candida albicans, in contrast to C. glabrata, is a polymorphic fungus that can grow as yeast or hyphae depending on the environmental conditions (Sudbery, 2011). The induction of germ tubes was unaffected by biotin pre-starvation or the availability of biotin, DTB or KAPA (Figure 1c). However, filament elongation was significantly reduced in medium lacking biotin or its precursors, as seen by reduced hyphal length after 4 hr with pre-starved C. albicans cells (Figure 1d) and reduced size of hyphal microcolonies after 24 hr (Figure 1e).

Mammalian cells can recycle biotin by proteolytic digestion of biotinylated proteins, releasing biocytin, which is further degraded to lysine and biotin (Zempleni, Hassan, & Wijeratne, 2008). To determine whether Candida spp. cells are able to utilise these potential host biotin sources, we analysed the growth of C. albicans and C. glabrata in the presence of biotinylated BSA or biocytin. Indeed, both species grew in the presence of biotinylated BSA or biocytin (Figure S1b).

Of note, biotin is not essential under all growth conditions. We found that the growth defect of C. glabrata and C. albicans in the absence of biotin was partly rescued when aspartate was added as a nitrogen source instead of ammonium sulfate (Figure S1c) or when casamino acids were present as the sole carbon and nitrogen source (Figure S3b,d). In fact, aspartate can be converted to the central metabolite oxaloacetate in a biotin-independent reaction (Yagi,
Kagamiyama, & Nozaki, 1982). This likely bypasses the need of the biotin-dependent pyruvate carboxylase for production of oxaloacetate by carboxylation of pyruvate (Ruiz-Amil, De Torrontegui, Palacian, Catalina, & Losada, 1965).

These data show that, under standard culture conditions, the presence of biotin, its precursors, or potential host biotin sources are essential for the growth of different pathogenic Candida species.

2.2 | C. glabrata and C. albicans genomes contain potential biotin-metabolic and regulatory genes

Biotin acquisition, biosynthesis, and regulation of these processes have been described in the model yeast Saccharomyces cerevisiae (Phalip, Kuhn, Lemoine, & Jeltsch, 1999; Stolz, 2009; Stolz, Hoja, Meier, Sauer, & Schweizer, 1999; Weider, Machnik, Klebl, & Sauer, 2006). To identify genes necessary for biotin acquisition and the maintenance of biotin homeostasis in the most frequently isolated Candida species, C. glabrata and C. albicans, we searched the genomes of these species for potential orthologs of S. cerevisiae biotin-related genes.

The biotin biosynthesis pathway was completely lost in the last common ancestor of Candida and Saccharomyces species, but has been partially rebuilt through horizontal gene transfer from bacterial species, resulting in the S. cerevisiae BIO2-5 incomplete biotin biosynthesis cluster which allows biotin synthesis from the precursor KAPA or DTB (Fitzpatrick, O'Gaora, Byrne, & Butler, 2010; Hall & Dietrich, 2007; Figure 2a). The C. albicans genome contains potential orthologs of the S. cerevisiae genes encoding the enzymes Bio2, Bio3 and Bio4 (CR_01930C, CaBIO2; CR_01920W, CaBIO3; CR_01910C, CaBIO4) (Fitzpatrick et al., 2010; Figures 2b and Figure S4). Although C. glabrata is more closely related to S. cerevisiae than to C. albicans, we did not identify potential Bio2-4 orthologs in this yeast, indicating a loss of biotin biosynthetic genes (Figure 2b). Orthologs of the potential KAPA and 7,8-Diaminopelargonic Acid (DAPA) transporter Bio5 (Phalip et al., 1999), however, were identified in both genomes (CR_01900C and CAGL0H03399g). The uptake of biotin or its precursor, DTB, is facilitated by the S. cerevisiae high-affinity H+-biotin symporter, Vht1 (vitamin H transporter 1) (Stolz et al., 1999). We found two putative C. glabrata orthologs (CAGL0K04609g, CAGL0K04565g), while the two best hits in C. albicans were CR_06790C and CR_06660W (Figure 2b). In addition, C. albicans CR_03270W has recently been described as a putative biotin transporter gene (Gaur et al., 2008). All potential orthologs contain 10–12 putative transmembrane helices, a feature common in plasma membrane transporters (Sonnhammer, von Heijne, & Krogh, 1998). To narrow down potential functional orthologs of biotin or biotin precursor transporter genes, we analysed gene expression in C. albicans and C. glabrata at different biotin levels.
Of the potential ScVHT1 orthologs, only the C. glabrata gene CAGL0K04609g (CgVHT1) and the C. albicans gene CR_03270W (CaVHT1) showed clear biotin-dependent expression (Figure S2). In addition, the potential C. albicans ortholog (CR_01900C, CaBIO5), but not the C. glabrata ortholog of ScBIO5, was induced upon biotin limitation (Figure S2). In S. cerevisiae, the expression of genes associated with biotin metabolism is induced by the transcriptional activator Vhr1 in response to low biotin (Weider et al., 2006). We found one potential ScVHR1 ortholog in C. glabrata and C. albicans—CAGL0M12496g (CgVHR1) and CR_00630W (CaVHR1), respectively (Figure 2b). Both orthologs share a highly conserved N-terminal putative DNA-binding domain and a C-terminal putative activation domain with their S. cerevisiae ortholog (Weider et al., 2006). CAGL0M12496g is currently annotated as a pseudogene with a premature translational stop codon (http://www.candidagenome.org/). Sequencing of CAGL0M12496g in C. glabrata genomic DNA revealed a frameshift in comparison to the annotated sequence (four cytosines at ORF position 473–476 in the annotated sequence [Skrzypek et al., 2017], but only three cytosines in our strain, see Appendix S2). The predicted protein deduced from our sequence data lacks the early translational stop codon and has a similar length as the S. cerevisiae ortholog (689 and 640 amino acids, respectively; Appendix S2). Finally, the biotin protein ligase Bpl1 is needed for the transfer of biotin to biotin-accepting proteins in baker’s yeast. The genes CAGL0I03806g (CgBPL1) and orf19.7645 (CaBPL1) represent potential orthologs in C. glabrata and C. albicans.

### 2.3 VHT1 is essential for biotin-dependent growth of C. glabrata and C. albicans

To test whether CgVHT1 and CaVHT1 have a role in biotin acquisition, we generated deletion strains of the respective genes by targeted mutagenesis. Indeed, these strains were unable to grow in the presence of intermediate biotin levels (2 μg/L biotin) and required high amounts (2 mg/L) of this vitamin for growth comparable to that of the wildtype. A delayed growth was observed with elevated biotin concentrations (0.2 mg/L). The growth defect was restored after re-introduction of either CgVHT1 or CaVHT1 (Figure 3a,b). Similarly, C. albicans hyphal induction and extension after 2, 4 and 24 hr was significantly reduced in the Cavht1 Δ/Δ mutant as compared to the wildtype in medium containing 2 μg/L or 0.2 mg/L of biotin (Figure 3c–e). Again, high amounts of biotin or reintegration of CaVHT1 were able to restore wildtype-like filamentation. These data strongly indicate that CgVHT1 and CaVHT1 indeed encode biotin importers.

### 2.4 Vhr1 is a transcriptional regulator of biotin-metabolic genes in C. glabrata and C. albicans

To elucidate whether CgVhr1 and CaVhr1 regulate biotin-related genes in C. glabrata and C. albicans, we analysed gene expression of potential target genes under biotin limitation (no biotin or 0.2 μg/L biotin) as compared to intermediate biotin levels (2 μg/L biotin) in wildtype strains and mutants lacking CgVHR1 and CaVHR1. Both,
**FIGURE 3**  VHT1 is essential for biotin-dependent growth. Yeast growth of biotin pre-starved wt, mutant (vht1Δ), or complemented strain (vht1Δ + VHT1) of (a) *Candida glabrata* (37°C) or (b) *Candida albicans* (30°C) in minimal medium containing 2 μg/L, 0.2 or 2 mg/L biotin. (c–e) Hyphal growth of non-starved or pre-starved *C. albicans* cells in RPMI1640 containing 2 μg/L, 0.2 or 2 mg/L biotin at 37°C and 5% CO2. (c) Percentage of germ tube-forming cells at 2 hr. (d) Hyphal length at 4 hr (mean ± SD of individual hyphae from three replicates). (e) Microcolony size at 24 hr, as a measure for long-term hypha formation (each single dot represents one individual microcolony). Values are represented as mean ± SD (a,b) or mean ± SD (c–e) of at least three replicates. For statistical analysis, a one-way ANOVA with Bonferroni’s multiple comparison test was used (**p ≤ .001 comparing wt and VHT1 deletion mutant; ###p ≤ .001 comparing VHT1 deletion mutant to VHT1 complemented strain).
CgVHT1 and CgBPL1 were induced upon biotin limitation in wildtype or the CgVHR1-complemented strain while CgVHR1 itself was steadily expressed independent of the biotin levels. The up-regulation of CgVHT1 and CgBPL1 was almost abolished in the CgVhr1Δ mutant (Figure 4a).

Similarly, C. albicans’ incomplete biotin biosynthesis cluster genes CaBIO2-5, as well as CaVHT1, CaBPL1 and CaVHR1 showed the highest expression under biotin-limiting conditions in wildtype cells and the CaVHR1-complemented strain (Figure 4b). Expression of CaBIO3, CaBIO4 and CaBIO5 was strongly reduced in the absence of CaVHR1, while CaVHT1 and CaBIO2 expression was unaffected by CaVHR1 deletion under biotin-limiting conditions and even increased in the presence of 2 μg/L biotin. CaBPL1 showed a mixed expression pattern with slightly reduced expression in the Cavhr1Δ/Δ mutant under biotin limitation, but higher expression in the presence of 2 μg/L biotin.

These data show that both CgVhr1 and CaVhr1 are important for the fungal response to environmental biotin availability. While CgVhr1, similar to its S. cerevisiae ortholog, activates genes associated with biotin import and intracellular transfer to proteins upon biotin limitation, CaVhr1-dependent activation seems to be limited to the biotin biosynthesis genes CaBIO3-5. Meanwhile, biotin uptake in C. albicans is reduced and depends on CaVhr1 when sufficient environmental biotin is available.

C. albicans and C. glabrata mutants lacking CgVHR1 and CaVHR1 were further tested for growth under biotin limitation (0.2 μg/L biotin). In contrast to the deletion of VHT1, the deletion of VHR1 did not lead to growth or hyphal defects under biotin limitation (Figure S3). In host

**FIGURE 4** VHR1 regulates biotin-metabolic genes. Non-starved (a) Candida glabrata or (b) Candida albicans cells were cultivated in minimal medium with indicated biotin concentrations for 90 min (C. glabrata) or 180 min (C. albicans) at 37°C. Target gene expression was analysed by qRT-PCR and normalised to CgACT1, CgEFB1, CgEFT2 and CaTDH3, CaEFT2. Values are represented as mean + SD of three independent experiments, whereby n-fold expression for each gene is shown relative to the wt in 2 μg/L biotin. For statistical analysis, a two-way ANOVA with Bonferroni post-tests was used (*p ≤ .05, **p ≤ .01, ***p ≤ .001; comparing wt and VHR1 deletion mutant; #p ≤ .05, ##p ≤ .01, ###p ≤ .001; comparing VHR1 deletion mutant to VHR1 complemented strain; n.d., not detectable)
niches like the macrophage phagosome, Candida cells likely do not encounter their preferred carbon and nitrogen sources in high amounts, but rather alternative sources like amino acids (Kaur et al., 2007; Lorenz, 2013; Lorenz et al., 2004). Therefore, we combined biotin limitation with the presence of casamino acids as the sole carbon and nitrogen source, instead of the optimal nutrients glucose and ammonium sulfate. Under these conditions, the Cgvrh1Δ/Δ mutant grew less than the wildtype (Figure S3b), whereas the Cavhr1Δ/Δ mutant grew similarly to the wildtype (Figure S3g). This suggests that C. glabrata requires CgVHR1 for growth under conditions that combine low biotin with limitations of optimal C- and N-sources.

2.5 The fungal biotin pool influences intracellular fitness inside macrophages

Metabolic plasticity is essential for the adaptation of Candida species to the changing environment of different, often nutrient-limited, host niches including the macrophage phagosome (Lorenz, 2013; Miramón & Lorenz, 2017). Both C. albicans and C. glabrata are able to survive macrophage phagocytosis and even replicate inside macrophages (C. glabrata, Seider et al., 2011) or form hypha and escape (C. albicans, McKenzie et al., 2010). Both species must have developed strategies to counteract macrophage killing activities, but also adapt to phagosomal nutrient conditions (Miramón et al., 2013). To elucidate how biotin impacts the fungal fitness inside macrophages, we first confronted biotin-starved (pre-incubation in medium without biotin) or biotin-fed (pre-incubation in medium with high biotin 2 mg/L) C. glabrata or C. albicans cells with primary human monocyte-derived macrophages (hMDMs). As a control, we used fungal cells pre-incubated in medium with intermediate biotin levels (2 μg/L) that are sufficient to promote growth. In macrophage confrontation assays, biotin pre-starvation increased the susceptibility of C. glabrata and C. albicans to phagocytosis-mediated killing (Figure 5a,c).

Moreover, biotin-starved C. glabrata cells were significantly reduced in intracellular replication in macrophages (Figure 5b) and biotin-starved C. albicans cells developed shorter filaments and showed reduced levels of hyphal outgrowth compared to fungal cells pre-cultivated with intermediate or high amounts of biotin (Figure 5d,e). To exclude that the observed effects were due to a
To address the general detrimental effect of vitamin limitation on fungus-macrophage interactions, we infected macrophages with \( C. \) \textit{glabrata} cells pre-starved for niacin (nicotinic acid), a vitamin needed for \( C. \) \textit{glabrata} to overcome NAD\(^+\) auxotrophy (Domergue et al., 2005). Nicotinic acid starvation did not reduce \( C. \) \textit{glabrata} survival inside macrophages (Figure 5f), indicating that the observed effect of biotin starvation is

**FIGURE 6**  Legend on next page.
specific. Up-regulation of biotin homeostasis and biosynthesis genes after phagocytosis by macrophages suggests that _C. glabrata_ and _C. albicans_ indeed experience biotin limitation inside macrophages (Figure 5g,h). Combined, these data indicate that biotin is crucial for survival and proliferation of _C. glabrata_ and _C. albicans_ in macrophages and that biotin availability for _C. glabrata_ and _C. albicans_ upon macrophage phagocytosis is limited.

### 2.6 VHR1 and VHT1 are essential for fungal fitness within macrophages

Next, we wanted to know whether disturbance of biotin uptake or biotin-dependent regulation would affect fungal fitness after macrophage phagocytosis. Therefore, we confronted hMDMs with biotin-fed _C. glabrata_ or _C. albicans_ VHT1 and VHR1 knockout strains and evaluated survival and intracellular proliferation of phagocytosed fungal cells (Figure 6).

The _C. glabrata_ Δvhr1 mutant, but not the _C. glabrata_ Δvht1 mutant, was significantly attenuated in intracellular survival as compared to the complemented strain or the wildtype (Figure 6a). Similarly, the _C. albicans_ Δvhr1ΔΔ, but not the _C. albicans_ Δvht1ΔΔ mutant, showed reduced survival rates. To rule out that stored biotin from the pre-culture compensates the need for VHT1 early after infection, we did parallel survival assays with pre-starved fungal strains (Figure S5). Indeed, pre-starved _C. glabrata_ Δvht1ΔΔ cells showed significantly lower survival rates than the wildtype. However, survival of the _C. glabrata_ Δvht1ΔΔ mutant was not reduced. In addition, a lower survival rate of vhr1Δ mutants was only observed when fungal cells were pre-grown in biotin-containing medium (Figure 6a,c), but not when biotin-starved fungal cells were used (Figure S5).

Even after biotin feeding, _C. glabrata_ intracellular replication was reduced for both the _C. glabrata_ Δvhr1Δ and _C. glabrata_ Δvht1Δ mutants (Figure 6b). Also, _C. albicans_ hyphae formed early after phagocytosis were shorter in both _C. albicans_ Δvhr1Δ and _C. albicans_ Δvht1Δ as compared to the wildtype or the complemented strain and both mutants showed reduced rates of hyphal outgrowth (Figure 6c–e).

While _C. glabrata_ can replicate inside macrophages without causing substantial damage (Seider et al., 2011), _C. albicans_ hyphal growth and other factors cause macrophage lysis within hours (Kasper et al., 2018; McKenzie et al., 2010; Vylkova & Lorenz, 2014). Therefore, we looked at macrophages confronted for 24 hr with _C. albicans_ and measured macrophage lactate dehydrogenase (LDH) release to determine host cell lysis. In addition, we quantified the sizes of _C. albicans_ microcolonies as a measure for sustained hyphal growth during contact with macrophages. While the _C. glabrata_ Δvhr1ΔΔ mutant formed smaller microcolonies and was severely attenuated in macrophage damage, the _C. glabrata_ Δvhr1ΔΔ showed wildtype-like microcolonies and cytotoxicity (Figure 6f,g).

Besides biotin, precursors like KAPA or DTB may support _C. albicans_ growth depending on its _BIO2-5_ partial biosynthesis cluster (see above). Such precursors may potentially be provided by co-inhabiting bacteria, for example, during gut colonisation, but are likely not available during direct _C. albicans_-host interactions, as these intermediates are not produced by host cells. We constructed a _C. albicans_ mutant lacking _CaBIO2-5_ and found that the _BIO2-5_ cluster is indeed necessary for growth with KAPA and DTB, but not biotin (Figure S4a). Intracellular survival, hyphal growth and damage of macrophages were unaffected in the _CaBIO2-5_ΔΔ mutant (Figure S4b–e), indicating that KAPA or DTB utilisation is dispensable for the _C. albicans_–macrophage interaction.

Collectively, these data indicate that in both _Candida_ species, Vhr1-regulated processes are important for short-term responses to macrophage antifungal activities and intracellular proliferation. Vht1-dependent biotin acquisition is needed for survival of _C. albicans_ cells that have experienced biotin limitation prior to phagocytosis. Vht1 also becomes relevant for _C. glabrata_ replication and early and sustained intracellular growth of _C. albicans_ hyphae and hypha-induced damage of macrophages.

### 2.7 The biotin transporter Vht1 is required for colonisation of the brain during systemic infection

Our _in vitro_ growth experiments show that both _C. albicans_ and _C. glabrata_ similarly require VHT1 for biotin acquisition, and mutants lacking VHT1 need at least x1,000 more biotin than wildtype cells for proliferation (Figure 3). To learn more about the availability of and requirement for biotin during _Candida_ infections, we analysed both...
Candida species in established systemic infection mouse models (Jacobsen et al., 2010; Jacobsen, Lüttich, Kurzai, Hube, & Brock, 2014). First, we infected mice via the tail vein with biotin pre-starved or biotin-fed C. glabrata wildtype, Cgvhr1Δ or Cgvht1Δ mutant, or the respective complemented strains. As expected from previous studies, systemic infection with C. glabrata caused no clinical symptoms and the only alteration upon necropsy was moderate splenomegaly, indicating an immune response to infection (Jacobsen et al., 2010) (not shown). Fungal burdens of infected organs were highest at Day 2 post-infection and decreased over time for all tested strains (Figures 7a and Figure S6). The biotin transporter mutant Cgvht1Δ was strongly attenuated in colonisation of the brain and caused less MCP-1 (monocyte chemoattractant protein 1; CCL-2) production, while the complemented strain showed wildtype-like behaviour (Figures 7a and Figure S7). In contrast, mice infected with the Cgvhr1Δ or Cgvht1Δ mutant showed wildtype-like fungal burdens and comparable cytokine levels in the kidney, liver and spleen (Figures S6a–c and S7). Since, according to our in vitro experiments,

**FIGURE 7** VHT1 is required for in vivo persistence of Candida glabrata and virulence of Candida albicans. (a) Five CD-1 mice per group were intravenously infected with biotin-starved C. glabrata wildtype (wt), mutant, or complemented strains (2.5 × 10⁷ yeast cells/20 g body weight in 100 μL PBS) on Day 0. The fungal burden of the brain was determined by quantifying cfu in tissue homogenates at indicated time points. (b–d) Balb/C mice were intravenously infected with biotin-starved C. albicans wildtype, mutant, or complemented strains (2 × 10⁵ yeast cells/20 g body weight in 100 μL PBS) on Day 0 and (b) survival of eight mice was followed up for 21 days. The fungal burden of (c) brain and (d) kidneys was evaluated by quantifying cfu in tissue homogenates of five mice at indicated time points. Statistical analysis was performed with a one-way ANOVA on datasets from the same day to compare the strains, followed by Dunn’s multiple comparison test. Survival curves were compared using the log rank (Mantel-Cox) test. (*p ≤ .05, **p ≤ .01, ***p ≤ .001 comparing wt and VHT1 deletion mutant; #p ≤ .05, ##p ≤ .01, ###p ≤ .001 comparing VHT1 deletion mutant to VHT1 complemented strain). Dashed lines represent the detection limit and dots below this line mean no cfu were detected.
CgVht1 in particular is essential for full fitness and proliferation in biotin-poor niches, we concluded that infecting CgVht1Δ cells may either find sufficient, accessible biotin for proliferation in these organs or that these cells persist in a non-proliferating stage. To provide proof that C. glabrata can remain viable in the absence of nutrients, we incubated the different C. glabrata strains in PBS without carbon, nitrogen or vitamin sources. The numbers of colony forming units (cfu) dropped over time, but stayed above 10% of the initial cell count, confirming that a fraction of C. glabrata cells can survive for 2 weeks, even in the absence of nutrients, and independent of CgVHR1 or CgVHT1 (Figure S6d).

Fungal burdens after infection with biotin pre-starved C. glabrata were slightly (but not significantly) increased as compared to non-starved C. glabrata in the liver, kidneys and brain on Day 2, but not on Day 7 and 14 (Figures 7a and S6a,c). Thus, biotin pre-starved C. glabrata may be slightly better adapted to early conditions during in vivo infection; however, the biotin status before infection has no substantial impact on fungal persistence. Secondly, we infected mice via the tail vein with biotin pre-starved C. albicans wildtype, Cavhr1Δ/Δ or Cavht1Δ/Δ mutant cells, or the respective complemented strains. Mouse morbidity was analysed by monitoring the development of clinical symptoms over a maximum of 21 days and mice were euthanized after reaching humane endpoints. The Cavht1Δ/Δ mutant was significantly attenuated in virulence, whereas the Cavhr1Δ/Δ mutant showed wildtype-like virulence (Figure 7b). Additionally, to gain insights into the organ-specific C. albicans fungal loads, a time course experiment analogous to the C. glabrata infection model was performed. We found that the Cavht1Δ/Δ mutant showed significantly lower fungal burdens in the brain and kidneys (Figure 7c,d), while those of the liver and spleen were comparable to the wildtype (Figure S6f,g). Correspondingly, the inflammatory response in the Cavht1Δ/Δ-infected brain and kidney but also liver was reduced, while the complemented strain induced a wildtype-like cytokine response (Figure S7).

Based on these data we conclude that Vhr1-dependent regulatory processes are dispensable for in vivo persistence of C. glabrata and virulence of C. albicans. However, Vht1-mediated biotin acquisition is required for persistence of C. glabrata in the mouse brain and virulence of C. albicans.

3 DISCUSSION

Candida albicans and C. glabrata are two successful opportunistic pathogenic fungi. These two yeast species are able to rapidly adapt to changing environments with varying nutrient availability (Miramón & Lorenz, 2017). Both C. glabrata and C. albicans are auxotrophic for the essential micronutrient biotin and, thus, must have found ways to acquire this vitamin during commensal growth and systemic infection (Ahmad Hussin et al., 2016; Firestone & Koser, 1960). On mucosal surfaces, C. albicans and C. glabrata live in close proximity to other microbes, which are often able to synthesise biotin and likely provide this vitamin or biosynthesis intermediates to colonising Candida cells (Hill, 1997). While C. albicans contains the incomplete biotin biosynthesis cluster BIO2-5, which enables this fungus to synthesise biotin when growing in the presence of KAPA or DTB (Figures 1, 2 and S4; Ahmad Hussin et al., 2016; Fitzpatrick et al., 2010). C. glabrata has apparently lost the biotin biosynthesis pathway (Figure 2) and cannot utilise these biotin precursors (Figure 1). This underlines the remarkable metabolic flexibility of C. glabrata, which can thrive in the host despite loss of several metabolic genes and resulting auxotrophies, such as NAD⁺, pyridoxine and thiamine (Brunke & Hube, 2013; Dujon et al., 2004; Kaur, Domergue, Zupancic, & Cormack, 2005). Presence or absence of BIO2-5 genes in other medically important yeasts (Fitzpatrick et al., 2010) similarly correlated with their ability to utilise biotin precursors (Figure S2): C. tropicalis (possessing BIO2-5), grew on KAPA and DTB while C. dublinensis and C. auris (possessing BIO2 only) grew on DTB and C. parapsilosis (lacking BIO2-5) was unable to utilise either biotin precursor. The BIO2-5 cluster may therefore be an advantage for C. albicans (and C. tropicalis) over C. glabrata (and C. dublinensis, C. auris and C. parapsilosis), allowing for better host colonisation in the presence of bacteria. Therefore, these genes may be seen as commensal factors. However, these genes are likely dispensable during systemic infection, as mammals are biotin auxotrophs and provide no biosynthesis intermediates. In agreement, BIO2-5 was dispensable for C. albicans–macrophage interaction (Figure S4).

The ability of C. albicans to undergo a morphological transition between yeast and hyphae is considered to be a main virulence attribute of this fungus (Jacobsen et al., 2012). Our data show that biotin is necessary for in vitro proliferation of C. glabrata and C. albicans yeast cells, but also for C. albicans hyphal growth (Figure 1). These data are supported by a previous study (Ahmad Hussin et al., 2016), which showed that biotin enhances C. albicans germ tube formation.

We have identified two major players of biotin homeostasis in C. albicans and C. glabrata—the ortholog of the S. cerevisiae biotin transporter Vht1 and the ortholog of the S. cerevisiae regulator of biotin-metabolic genes Vhr1. As mutants lacking VHT1 were unable to grow in vitro unless high amounts of biotin were present and as VHT1 expression was highly up-regulated in medium with low biotin in both Candida spp., we conclude that Vht1 is the main factor facilitating biotin acquisition in both, C. albicans and C. glabrata (Figures 3, 4 and S2). Genomes of other biotin-auxotrophic Candida spp. such as C. tropicalis, C. dublinensis, C. auris and C. parapsilosis, also contain potential VHT1 orthologs (Skrzypek et al., 2017), indicating that the biotin uptake machinery is conserved among other medically important Candida species.

Our data show that Vhr1 regulates the expression of biotin-metabolic genes, including VHT1 in C. albicans and C. glabrata. While C. glabrata Vhr1 induces CgVHT1 expression upon biotin limitation, C. albicans Vhr1 was required for keeping CaVHT1 expression low under biotin-replete conditions (Figure 4). This suggests that a yet unknown regulator activates CavHT1 expression in C. albicans. A Cgvr1Δ mutant was still able to express VHT1 to some extent, suggesting the presence of additional regulators in C. glabrata as well. This may also explain why mutants lacking VHR1 were still able to grow under biotin-limiting conditions (Figure S3) or showed
wildtype-like fitness *in vivo* (Figures 7 and S6). In contrast, a Cgvr1Δ mutant was outcompeted by the wildtype in a previous infection study, suggesting an involvement of CgVHR1 in *in vivo* fitness (Brunke et al., 2015). However, that mutant was generated in a strain background with amino acid auxotrophies. Such auxotrophies alone have no impact on *C. glabrata* fitness in the mouse (Jacobsen et al., 2010), but may be detrimental when combined with the absence of Vhr1-dependent regulation. Our data, however, show that VHR1 is required for survival and proliferation in macrophages (*C. albicans* and *C. glabrata*) as well as growth when biotin limitation is combined with the replacement of glucose by casamino acids (Figure S3). These data suggest that Vhr1-dependent regulation becomes important in specific niches, when biotin limitation is combined with the limitation of other nutrients and/or antimicrobial activities of immune cells (see below).

Our data obtained from macrophage confrontation assays implies that the *Candida*-containing phagosome is limited in biotin, as biotin-pre-starvation reduced *Candida* survival, as the biotin transporter gene VHT1 was up-regulated in both *Candida* species upon biotin pre-starvation and after phagocytosis, and as deletion of VHT1 led to slower replication of *C. glabrata* and slower hyphal extension of *C. albicans* inside macrophages (Figures 3, 5, 6 and S5). This concept of biotin limitation inside the phagosome is supported by studies in bacterial pathogens like *Francisella* spp. and *Mycobacterium* spp., which require a functional de novo biotin biosynthesis pathway for intracellular fitness within macrophages (Feng et al., 2015; Napier et al., 2012; Rengarajan, Bloom, & Rubin, 2005; Yu et al., 2011). Correlating with the reduction in hyphal formation of a Cavht1Δ/Δ mutant during the course of macrophage interaction, we observed a reduction in macrophage damage—a phenotype confirmed by a previous study (O’Meara et al., 2018).

Vhr1 was similarly important for replication (*C. glabrata*) or early hypha formation in macrophages (*C. albicans*), but not for sustained *C. albicans* filamentation and late macrophage damage. Of note, a Cavhr1Δ/Δ mutant exhibited wildtype-like VHT1 expression in macrophages (not shown). In addition, VHR1 was only necessary for *Candida* survival under conditions were VHT1 was dispensable (when biotin was high before phagocytosis, Figure S5). These data suggest that CoVhr1 functions during early stages of macrophage interaction are independent of Vht1 regulation and become dispensable at later stages. Instead, CoVht1-dependent biotin uptake is needed for sustained proliferation inside macrophages. In *C. glabrata* we observe reduced CgVHT1 expression in the absence of CgVHR1 (not shown), which likely limits biotin import and consequently replication after phagocytosis.

Together, our macrophage confrontation data suggest that Vht1-dependent biotin uptake is required for sustained proliferation of *C. glabrata* and *C. albicans* inside macrophages, while initial fitness depends on VHT1-independent Vhr1 functions in fungal survival.

Our main conclusions on the importance of biotin during *Candida* infection are based on *in vivo* data with *C. glabrata* and *C. albicans* vht1Δ mutants, which are unable to synthesise and acquire biotin unless high amounts are present (Figure 3). Our data clearly show that Vht1 is essential for the full virulence of *C. albicans* (Figures 7 and S6). Fungal organ burdens, however, were reduced in the kidney and brain, but not in the spleen or liver. Similarly, *C. glabrata* persistence in mice was not generally reduced upon VHT1 deletion (Figures 7 and S6). In fact, VHT1 deletion mutants of *C. glabrata* showed wildtype-like fungal burden in the kidney, liver, and spleen and reduced cfu numbers were only observed in mouse brains. These data indicate that biotin availability is high enough to support *Candida* growth in organs like the liver and spleen, while access to biotin is limited in the brain. In line with this, avidin-based histology staining suggested that hepatocytes and proximal tubular epithelial cells in the kidney contain high amounts of biotin (Combs & McClung, 2017; Cooper, Kennedy, McConnell, Kennedy, & Frigg, 1997), while biotin levels in the extracellular space of the brain tissue are lower (8 nM in rabbit [2 μg/L]) (Spector & Johanson, 2007). Such levels would be sufficient to promote growth of Candida wildtype cells, but not VHT1 deletion strains (Figure 3). An alternative explanation is that certain nutrients, like aspartate or other amino acids, may bypass the need for biotin in certain host niches as suggested by our *in vitro* data (Figures S1 and S3).

Of note, biotin levels available in humans may be substantially lower than in mice, as a recent study showed a 40-fold higher biotin concentration in mouse plasma compared to human plasma (Carfrae et al., 2020). In line with this, the same study showed that growth of bacterial pathogens, which lack biotin transporters and are deficient in biotin biosynthesis, is diminished in human plasma, but not mouse (Carfrae et al., 2020).

Biotin may become dispensable for colonising *Candida* cells in cases of weak or no fungal metabolic activity or proliferation. Fungal burdens of *C. glabrata* in brains, but not kidney, liver, or spleen of mice increase between 12 hr and 3 days post-infection, which may indeed point to *C. glabrata* replication in the brain, but not in the other tested organs soon after infection (Beyer et al., 2018). This exciting possibility of non-proliferating *C. glabrata* cells in the host may also contribute to the high tolerance of *C. glabrata* towards fungistatic antifungal drugs. Similarly, increases of *C. albicans* fungal burdens in mouse kidney and brain, but not in liver or spleen (Lionakis, Lim, Lee, & Murphy, 2011) point to replication in those organs in which we observed reduced cfu of the Cavht1Δ/Δ mutant.

Collectively, our data show for the first time that Vht1-dependent fungal biotin acquisition is linked with intracellular proliferation of the opportunistic fungal pathogens *C. albicans* and *C. glabrata* in macrophages and in *in vivo* persistence of *C. glabrata* and virulence of *C. albicans* in mice. Our results underline the importance of micro-nutrients, such as biotin, for fungal pathogens.

### 4 | Experimental Procedures

#### 4.1 | Ethics statement

Human blood was taken from healthy volunteers with written, informed consent. The blood donation protocol and use of blood for
this study were approved by the institutional ethics committee of the university hospital Jena (Ethik-Kommission des Universitätsklinikums Jena, Permission No. 2207–01/08). Animal experiments were performed in compliance with the European and German animal protection laws and were approved by the ethics committee "Beratende Kommission nach §15 Abs. 1 Tierschutzgesetz" and the responsible Federal State authority Thüringer Landesamt für Verbraucherschutz. Bad Langensalza, Germany (Permit No. 03-026/16, HKI-19-005). All mice were cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

4.2 Strains and general growth conditions

All strains used in this study are listed in Table S1. Escherichia coli was cultivated in liquid lysogeny broth with 50 μg/ml ampicillin for plasmid generation. For growth on solid medium, 2% agar was added. Candida strains were pre-cultured overnight in liquid Yeast Peptone Dextrose (YPD) broth (2% glucose, 2% peptone and 1% yeast extract) at 30 or 37°C, with shaking at 180 rpm for 14–16 hr. The biotin concentration in standard YPD is 10–20 μg/L (Ogoshi et al., 2014 and own observations). As gene deletion of VHT1 led to strong growth defects in standard media, we routinely pre-cultured strains for experiments including the biotin transporter mutant vht1Δ in YPD + 2 mg/L biotin. Stationary phase yeast cells from pre-cultures were washed three times in PBS and the cell number was adjusted in PBS or culture medium.

For biotin and niacin pre-starvation, the YPD-grown, PBS-washed cell suspension was adjusted to an OD of 0.2 in minimal medium either without biotin (0.69% yeast nitrogen base [YNB] without biotin, amino acids, with ammonium sulfate [Formedium]; 2% glucose) or without niacin (0.69% YNB without niacin, amino acids, with ammonium sulfate [Formedium]; 2% glucose) and incubated for further 24 hr at 30 or 37°C, with shaking at 180 rpm for 14–16 hr. The biotin concentration in standard YPD is 10–20 μg/L (Ogoshi et al., 2014 and own observations). As gene deletion of VHT1 led to strong growth defects in standard media, we routinely pre-cultured strains for experiments including the biotin transporter mutant vht1Δ in YPD + 2 mg/L biotin. Stationary phase yeast cells from pre-cultures were washed three times in PBS and the cell number was adjusted in PBS or culture medium.

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4.3 Growth assays

Twenty microliters of a yeast cell suspension (5 × 10⁶ cells/ml) were added to 180 μl media in a 96-well plate (Tissue Culture Test Plate, TPP Techno Plastic Products AG). The growth was monitored by measuring the absorbance at 600 nm every 30 min for 100 cycles at 30 or 37°C using a Tecan Reader (Plate Reader infinite M200 PRO, Tecan Group GmbH) with orbital shaking (30 s, amplitude: 6 mm, wait: 10 s) before each measurement and multiple reads per well. All experiments were done in technical duplicates and, at least, biological triplicates.

Filamentation of C. albicans was induced in biotin-free RPMI1640 medium (PAN-Biotech) with L-glutamine (Thermo Fisher Scientific) either without biotin or with 2 μg/L of biotin/KAPA/DBT at 37°C and 5% CO₂ in a 24-well plate. Briefly, 5 × 10⁴ cells/well were incubated for 2 and 4 hr to allow formation of short hyphae. In parallel, to measure long hyphae in vitro, 100 cells/well were incubated for 24 hr to generate microcolonies originating from single yeast cells that formed radially branching filamentous hyphae (McCall, Kumar, & Edgerton, 2018). Following this, cells were fixed by adding one-tenth of the volume of 37% paraformaldehyde. Microscopy images were recorded using the Zeiss Axio Vert.A1 (Carl Zeiss Microscopy). Germ tube formation (2 hr) and hyphal length (4 hr) were measured for at least 200 cells/sample and the diameter of at least 10 microcolonies was determined (both in at least three replicates).

4.4 C. glabrata mutant generation

For construction of C. glabrata vhr1Δ and vht1Δ mutants, the upstream and downstream homologous regions of CAGL0M12496g (VHR1) and CAGL0K04609g (VHT1) were first subcloned with flanking restriction enzyme recognition sites (5’ Sphl- and 3’ Saci for upstream region of VHR1, 5’ Sphl and 3’ Ncol for upstream region of VHT1, 5’ Spel- and 3’ SacI for downstream regions of VHR1 and VHT1) using the TOPO Cloning Kit (Invitrogen). The homologous regions were then excised and cloned into the plasmid pTS50 (Schwarzmüller et al., 2014) to generate cassettes containing the NAT1 selection marker (Schwarzmüller et al., 2014; Table S3) surrounded by gene-specific regions. Integration was confirmed by restriction digestion. Deletion constructs were amplified from generated plasmids using P1 and P4 primers (Table S2) and purified with QiAquick PCR purification kit (Qiagen). Next, 4 μg of the PCR product was transformed into the parental strain wildtype strain ATCC2001 or a cryptophan auxotrophic derivative (trp1Δ) using a modified heat-shock method (Sanglard, Ischer, Monod, & Bille, 1996 with heat shock: 15 min at 45°C). Mutants were selected for on YPD agar plates with 250 μg/ml nourseothricin. Growth of vht1Δ clones was facilitated by adding 2 mg/L biotin to selective agar plates.

Complemented strains were generated by reintegrating VHR1 and VHT1 into the TRP1 locus. This locus was chosen as tryptophan auxotrophy (trp1Δ) had no negative impact on C. glabrata in vivo fitness and in vitro mutant growth phenotypes (Jacobse et al., 2010; Yanez-Carrillo et al., 2015). In detail, the TRP1 gene and its 5’ upstream region (~984 bp to +750 bp) was PCR-amplified with primers introducing flanking Sphl restriction sites and ligated into the Sphl-linearized pUC19 vector (In-Fusion HD Cloning Kit) to generate pUC19-TRP1. Subsequently, the TRP1 3’ downstream region (~751 bp to +1,570 bp) was PCR-amplified with primers introducing XmaI and SacI restriction sites and was ligated into the XmaI/Sacl opened plasmid pUC19-TRP1 to create the final pTRP1. Plasmids pTRP1-VHR1 and pTRP1-VHT1 were generated by PCR amplifying the coding sequences of VHR1 and VHT1 together with the up- and downstream intergenic regions with primers introducing 15 bp flanks of the empty pTRP1 (VHR1) or Sar and Xbal restriction sites (VHT1). Constructs were cloned into Xbal-digested pTRP1
CD14-positive monocytes were selected for by automated cell sorting. Separation Media (Capricorn Scientific) in Leucosep tubes (Greiner) were donated by healthy volunteers were separated through Lymphocytes (to generate pTRP1-VHR1 or pTRP1-VHT1) cassettes were amplified by PCR, purified and transformed into a trplΔ background strain to generate tryptophan prototrophic mutants or complemented strains, respectively. Resulting clones were selected for on solid minimal medium. All deletions and re-integrations were confirmed by PCR and Southern blot.

4.5 C. albicans mutant generation

_Candida albicans_ vhr1Δ/Δ (CR_00630WΔ/Δ) and vht1Δ/Δ (CR_03270WΔ/Δ) mutants were generated with a gene disruption method (Wilson, Davis, & Mitchell, 1999) and lithium acetate transformation protocol (Walther & Wendland, 2003). Briefly, the Arg-, His- and Ura auxotrophic parental strain BW217 was sequentially transformed with 10 μg of PCR-amplified and purified His1 and Arg4 deletion cassettes, which were flanked by 100 bp of the target homology region, resulting in disruption of the open reading frames of both alleles of CR_00630W (VHR1) or CR_03270W (VHT1). Primers used for PCR amplification and verification are listed in Table S2. Growth of vht1Δ/Δ clones was facilitated by adding 2 mg/L of biotin to selective agar plates. Homozygous uridine auxotrophic deletion mutants were then transformed with the Stul-linearized empty Clp10 plasmid (Murad, Lee, Broadbent, Barell, & Brown, 2000) to generate prototrophic mutants or with Stul-linearized Clp10-VHR1 or Clp10-VHT1 plasmids to generate complemented strains. For the generation of Clp10-VHR1 or Clp10-VHT1, the coding sequences of VHR1 as well as VHT1 together with their native up- and downstream intergenic regions were cloned into Clp10 using KpnI and SalI (Clp10-VHR1) or XhoI (Clp10-VHT1). Constructed plasmids were verified by sequencing and the correct integration of deletion and complementation cassettes was confirmed by PCR and Southern blot.

4.6 Isolation and differentiation of hMDMs

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 labelled CD14-positive monocytes were selected for by automated cell sorting (autoMACS; MiltenyiBiotec). To differentiate monocytes into adherent hMDMs, 1.7 × 10^7 cells were seeded into 175 cm^2 cell culture flasks in RPMI1640 media with L-glutamine (Thermo Fisher Scientific) containing 10% heat-inactivated foetal bovine serum (FBS; BioSSELL) and 50 ng/ml recombinant human macrophage colony stimulating factor (M-CSF) (Immunotools) and incubated for 5 days at 37°C and 5% CO2 until the medium was exchanged. After another 2 days, adherent hMDMs were detached with 50 mM EDTA in PBS and seeded in 6-well plates (1 × 10^4 hMDMs/well), 12-well plates (4 × 10^5 hMDMs/well), 24-well plates (2 × 10^5 hMDMs/well) or 96-well plates (4 × 10^4 hMDMs/well) in RPMI with 50 ng/mL M-CSF and incubated over-night. Prior to macrophage infection, medium was exchanged with serum- and biotin-free RPMI medium with l-glutamine (Thermo Fisher Scientific) to exclude biotin uptake by Candida prior to phagocytosis.

4.7 Macrophage killing assay

Fungal survival was quantified as described previously (Seider et al., 2014). Briefly, macrophages in 24-well plates were stimulated for at least 24 hr with 20 ng/mL of recombinant human IFN-γ (Immunotools) in RPMI containing 50 ng/mL M-CSF and 10% FBS (see above) to favour a pro-inflammatory macrophage phenotype. Intracellular fungal survival was evaluated after 3 hr of coincubation with _C. glabrata_ or _C. albicans_ at a multiplicity of infection (MOI) of 1. Extracellular _Candida_ cells were removed by washing three times with PBS and intracellular yeast cells were released by lysing the macrophages with 0.1% Triton-X-100 for 10 min. Appropriate dilutions were plated in duplicate on YPD plates containing 2 mg/L of biotin and incubated for 1–2 days at 37°C. Colonies were counted with an automated colony counter ProtoCOL3 (Synbiosis, Cambridge, UK). To correct for inoculation differences between strains, colony numbers determined from intracellular lysates were normalised to colony numbers from plated culture inocula before calculating survival.

In experiments investigating the influence of niacin, hMDMs were seeded in 96-well plates and stimulated for at least 24 hr with 5 ng/mL of recombinant human IFN-γ (Immunotools) in RPMI1640 medium containing 50 ng/mL M-CSF and 10% FBS. Before confrontation with yeast cells, macrophages were washed with RPMI1640 medium without FBS and niacin. Niacin-free RPMI1640 medium was self-made and contains l-glutamine, phenol red, and sodium bicarbonate, but lacks niacinamide. The medium was adjusted to pH 7.2 and filter sterilised. Intracellular fungal survival was evaluated after 3 hr of coincubation with _C. glabrata_ at an MOI of 1 in technical triplicates. The following steps were performed as described above.

4.8 Differential staining after macrophage infection

Macrophages (1.5 × 10^5 hMDMs/well) were allowed to adhere onto coverslips in a 24-well plate overnight in RPMI containing 50 ng/mL M-CSF and 10% FBS and infected with _C. albicans_ at an MOI of 2 for 10 hr. Phagocytosis was synchronised on ice for 30 min followed by two washing steps with pre-warmed medium to remove unbound _Candida_ cells. The staining protocol was adapted from Kasper et al. (2018). Briefly, infected macrophages were fixed with Roti-HistoFix 4% and stained with 50 μg/mL Canavanalin A conjugated with Alexa Fluor 647 (ConA-647; Thermo Fisher Scientific) at 37°C for 30 min to visualise external _Candida_ cells. After permeabilisation with 0.5% Triton-X-100, extracellular and intracellular fungal cells were stained with 35 μg/mL Calcofluor White. Coverslips were mounted with Mowiol mounting medium and fluorescence images were recorded using the
Zeiss AXIO Observer.Z1 (Carl Zeiss Microscopy). Outgrowth rates of intracellular hyphae and hyphal length of internalised C. albicans were calculated by manually counting a minimum of 100 yeast cells/sample.

4.9 | Macrophage damage assay

Macrophages were allowed to adhere in 96-well plates overnight in RPMI containing 50 ng/mL M-CSF and 10% FBS and infected in technical triplicates with C. albicans at an MOI of 2 for 24 hr at 37°C and 5% CO₂. Macrophage lysis was determined by measuring the release of LDH into the cell culture supernatant using the Cytotoxicity Detection kit (Roche). A low (non-infected cells) and a high control (cells lysed with 0.2% Triton-X-100) were performed to calculate the percentage of cytotoxicity. In the same samples, the diameter of microlysed cells was measured to determine C. albicans hyphal length after 24 hr (≥10 colonies per sample).

4.10 | Determination of intracellular replication in macrophages

The protocol was adapted from Dagher et al. (2018) with minor changes. Prior to infection, C. glabrata cells were stained with 0.2 mg/mL fluorescein isothiocyanate (FITC) in carbonate buffer (0.15 M NaCl, 0.1 M Na₂CO₃, pH 9.0) for 30 min. Afterwards, yeast cells were washed three times in PBS and macrophages were infected at an MOI of 2 for 20 hr. To evaluate intracellular replication, macrophages were washed three times with PBS and lysed with 0.5% Triton-X-100 for 10 min. Released yeast cells were washed once with PBS, then with 2% BSA in PBS, and counterstained with 50 μg/mL ConA-AF647 in PBS at 37°C for 30 min. The ConA-AF647-stained yeast cells were washed two times with PBS and fixed with RotiHistofix 4% (Roth) for at least 20 min at room temperature. The ratio of FITC-positive and -negative yeast cells was evaluated with BD FACS Verse (BD Biosciences, Franklin Lakes) counting 10,000 events. Data analysis was performed using the FlowJO 10.2 software (FlowJo LLC, Ashland). The gating strategy was based on the detection of single, ConA positive cells and exclusion of cellular debris. The assessment of intracellular replication with FACS allows the relative distinction between mother and daughter cells.

4.11 | Fungal RNA isolation

For preparation of RNA from in vitro cultured Candida cells, stationary phase yeast cells were washed three times in PBS and 2 × 10⁷ Candida cells/mL were inoculated into minimal medium with different biotin concentrations. At indicated time points, cells were harvested and centrifuged (4,000g, 10 min and 4°C). The cell pellet was washed with ice-cold water, centrifuged again, and immediately frozen in liquid nitrogen. For preparation of RNA from phagocytosed Candida cells, stationary phase yeast cells were washed three times in PBS. Macrophages (1 × 10⁶ hMDMs/well) were infected at an MOI of 10 in serum and biotin-free RPMI (PAN Biotech) with L-glutamine (Thermo Fisher Scientific). Phagocytosis was synchronised for 30 min on ice and unbound Candida cells were removed by two washing steps with warm RPMI medium. After 3 hr of incubation at 37°C and 5% CO₂, infected macrophages were washed three times with ice-cold PBS and lysed with 750 μL RLT buffer (Qiagen) containing β-mercaptoethanol. Phagocytosed fungal cells were collected by using a cell scraper to loosen the host cells and centrifuged (20,000g, 12 min and 4°C). The fungal cells were washed once with RLT buffer to remove most of the host RNA and immediately frozen in liquid nitrogen.

The isolation of the fungal RNA was performed as previously described (Lüttich, Brunke, & Hube, 2012). The fungal RNA from infected host cells was treated with 3 U Baseline-ZERO DNase (Biozym) in a total volume of 50 μL at 37°C for 45 min. The RNA was then precipitated by adding 1 volume of isopropanol 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).

4.12 | Expression analysis by reverse transcription-quantitative PCR

DNase-treated RNA (600 ng) was transcribed into cDNA using 0.5 μg of oligo-dT₁₂₋₁₈. 100 U Superscript III Reverse Transcriptase and 20 U RNaseOUT Recombinant RNase Inhibitor (all: Thermo Fischer Scientific) in a total volume of 35 μL for 2 hr at 42°C followed by heat inactivation for 15 min at 70°C. The cDNA was diluted from 1:5 to 1:20 in Diethyl pyrocarbonate (DEPC)-treated water and used for quantitative PCR with EvaGreen QPCR Mix II (BioS&SELL) performed in a CFX96 thermocycler (Bio-Rad). Primers (Table S2) were used at a final concentration of 500 nM. Target gene expression was calculated using the ΔΔCt method (Pfaffl, 2001), with normalisation to the housekeeping genes CgACT1, CgEFB1, CgEFT2 for C. glabrata or CaACT1, CaTDH3, CaeEFT2 for C. albicans.

4.13 | Mouse model of systemic C. glabrata infection

Female, specific-pathogen-free CD-1 mice (Charles River, Germany; 8–10 weeks, 20–22 g) were randomly assigned into groups of five upon arrival and housed in individually ventilated cages. All Candida strains were either pre-starved for or fed with biotin for 24 hr before infection. On Day 0, mice were intravenously challenged with 2.5 × 10⁷ cfu/20 g body weight in 100 μL PBS via the lateral tail vein. The health status was monitored at least twice a day. On days 2, 7 and 14, five mice per strain were euthanised with an overdose of ketamine (10 mg/20 g body weight) and xylazine (0.5 mg/20 g body weight) applied intraperitoneally.
4.14 | Mouse model of systemic C. albicans infection

Female, specific-pathogen-free BALB/cAnNrtj mice (Janvier, France; 8 weeks, 18–22 g) were randomly assigned into groups of four (survival) and five (kinetics) upon arrival and housed in individually ventilated cages for the course of the experiment. On Day 0, mice were intravenously challenged with biotin pre-starved 2 × 10^5 cfu/20 g body weight in 100 μL PBS via the lateral tail vein. The health status was monitored at least twice a day. Mice which reached the humane endpoints (corresponding to moderate suffering and determined by a scoring system based on body weight, general appearance, changes in posture and behaviour, and body surface temperature) were euthanised with an overdose of ketamine (10 mg/20 g body weight) and xylazine (0.5 mg/20 g body weight) applied intraperitoneally. To determine the kinetic of fungal burden and inflammatory response in different organs, groups of five mice per strain were sacrificed 24 and 72 hr post-infection as described above.

4.15 | Quantification of fungal burden and cytokine levels in infected tissues

The mouse spleen, liver, kidneys and brain were removed aseptically, weighed and homogenised in tissue lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 28 μg/ml apropin; pH 7.4) using an Ika T10 basic Ultra-Turrax homogeniser (Ika, Staufen, Germany). To determine fungal burden, serial dilutions of homogenates were cultured on YPD plates containing 2 mg/L biotin and 80 μg/mL chloramphenicol. Colonies were counted after 24–48 hr of incubation at 30–37°C with an automated colony counter ProtoCOL3 (Synbiosis, Cambridge, UK). The fungal burden was calculated as cfu per gram of tissue.

For cytokine profiling, tissue homogenates were immediately centrifuged (1,500 × g, 15 min, 4°C) and supernatants were stored at -80°C. For cytokine measurement, frozen tissue homogenates were thawed on ice and centrifuged once (1,500g, 15 min, 4°C). The supernatants were diluted in supplied assay diluent supplied by manufacturer and enzyme and cytokine levels were determined by commercially available mouse enzyme-linked immunosorbent assay (ELISA) kits (Interleukin 1β [IL-1β], monocyte-chemoattractant protein-1 [CCL-2 or MCP-1], Interleukin 6 [IL-6] and Interferon-γ [IFN-γ] ELISA Ready SET Go! eBioscience, Thermo Fisher Scientific and myeloperoxidase [MPO] DuoSet, R&D systems) according to the manufacturer’s instructions. Identified by BLAST search, based on S. cerevisiae protein sequences. Direct comparison of two sequences was done using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Transmembrane prediction analysis was done using TMHMM Server v. 2.0 (prediction of transmembrane helices in proteins, http://www.cbs.dtu.dk/services/TMHMM/) and TMpred (membrane-spanning region prediction, https://embnet.vital-it.ch/software/TMPRED_form.html; Hofmann & Stoffel, 1993). Data are reported as scatterplot with mean ± SD, line charts with mean ± SD, or bar charts showing mean ± SD. Data were analysed using GraphPad Prism 5 (GraphPad Software, San Diego) and a one-way analysis of variance (ANOVA) with Bonferroni’s or Dunn’s multiple comparison test. For statistical analysis of matched observations in macrophage experiments and experiments on wildtype filamentation, a repeated measures ANOVA with Dunnett’s or Bonferroni’s multiple comparison test was performed. To represent the scattering of hyphal length, we illustrate the mean and standard deviation of all measured hypha in three replicates, but have used the mean of each replicate for statistical analysis. Reverse transcription-quantitative PCR data were statistically analysed using a two-way ANOVA with Bonferroni’s post-tests to evaluate differences between the strains and biotin concentrations. Statistically significant results are marked with a single asterisk or hash meaning p ≤ .05, double asterisks or hashes meaning p ≤ .01 or triple asterisks or hashes meaning p ≤ .001, n.d., not detectable. If symbol/colour explanations apply to several graphs in a row, one legend is given for neighbouring sub-panels on the right.

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CONFLICT OF INTEREST

The authors have no conflict of interest.
AUTHOR CONTRIBUTIONS
M.S., L.K., K.G., I.D.J. and B.H. were involved in the conception or design of the study. M.S., T.S.H., S.A., S.W., M.J.N. and L.K. contributed to the acquisition, analysis, or interpretation of the data. M.S., L.K. and B.H. wrote the manuscript.

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