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Role of Amino Acid Metabolism in the Virulence of Human Pathogenic Fungi

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Abstract

Purpose of the Review The success of *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* as fungal pathogens depends on their rapid adaptation to host microenvironments, through metabolic remodeling, stress resistance, and expression of virulence determinants. Amino acids represent an abundant nitrogen and carbon source within the host; however, their acquisition by fungi is a very complex process that interconnects several sensory and uptake systems and downstream pathways. In this review, we will summarize the current knowledge concerning this topic, identify gaps or discrepancies, and discuss future research directions.

Recent Findings Aside from supporting basic cellular functions, the utilization of many amino acids has a direct effect on fungal pathogenicity by triggering key virulence traits, including hyphal morphogenesis and biofilm growth in *C. albicans*, capsule formation in *C. neoformans*, and melanization in *A. fumigatus*.

Summary Although many components of amino acid sensing and metabolism are fungal specific, their importance in infection and potential as candidates for antifungal drug development require further investigation.

Keywords Amino acid metabolism · *Candida* · *Cryptococcus* · *Aspergillus* · Virulence · Nutrient sensing

Introduction

Invasive fungal infections are an increasing human health problem, particularly among individuals with impaired immunity. The main agents causing these infections are the ascomycetes, *Candida albicans* (Ca) and *Aspergillus fumigatus* (Af), and the basidiomycete, *Cryptococcus neoformans* (Cn), which account for more than 1.5 million invasive infections per year with mortality rates between 20 and 95% [1]. Poor diagnosis, intrinsic or acquired antifungal resistance, and the limited number of available antifungal drugs are the main challenges in their clinical treatment.

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Besides the primary virulence factors, which have been studied more extensively, metabolic versatility represents an integral part of fungal pathogenicity, since it provides the platform for nutrient assimilation and adaptation to host-imposed stress and antifungal drugs [2–13]. For the pathogenic fungi reviewed here, metabolic adaptations have evolved dependent on their environmental reservoir, generating the basis for the differences in nutrient utilization. *C. albicans* is a commensal of humans where it colonizes several distinct host niches and has developed a remarkable metabolic plasticity that governs its survival within the host. In contrast to this tight association with humans, *C. neoformans* and *A. fumigatus* are ubiquitous environmental fungi that are commonly found in soil, compost, and bird guano, which enter the human via the accidental inhalation of spores. Despite the differences in their primary natural habitats, all three fungi have developed multiple mechanisms for fast and effective acquisition of amino acids, available in free form or as part of host proteins in every host niche [14–18]. Aside from supporting growth, amino acid metabolism can trigger virulence traits, including hyphal morphogenesis, biofilm formation, melanization, and capsule formation [19, 20•, 21•, 22•, 23•]. Importantly, pathogenic fungi possess multiple amino acid transporters and biosynthetic pathways

not present in humans and essential for fungal growth, pointing to their potential as drug targets [24]. In this review, we will highlight and critically discuss the latest findings in sensing, uptake and metabolism of amino acids, and their role in fungal virulence, and suggest future research perspectives. Given that the topic is best investigated in *C. albicans*, we will discuss this organism primarily and include interesting findings for *C. neoformans* and *A. fumigatus* where available.

Amino Acid Sensing and Regulation of Their Metabolism

Fungi have evolved several sensing mechanisms and regulatory circuits to monitor and utilize extracellular amino acids, like the nitrogen catabolite repression (NCR), the target of rapamycin (TOR) pathway, and SPS and transceptor-mediated amino acid sensing (Table 1). They are all well-studied in the model yeast *Saccharomyces cerevisiae* (Sc) and therefore used here as a reference point [36]. The role of NCR and TOR in pathogenic fungi was summarized recently and will be mentioned only briefly [37].

Many fungi preferentially utilize ammonium, glutamine, and glutamate as nitrogen sources, while nitrates, nitrites, purines, amides, other amino acids, and proteins are used when primary nitrogen sources are limited. The utilization of secondary nitrogen sources is directed by a global control mechanism named NCR and mediated by the GATA-type transcription factors ScGat1 and ScGln3, which positively regulate the expression of permeases and pathway-specific catabolic enzymes upon nitrogen starvation [36, 38, 39]. *S. cerevisiae* orthologs governing similar regulatory circuits are described for all three pathogenic fungi [27–30, 40, 41]. It has been demonstrated that CaGln3 is also required for nitrogen starvation–induced filamentation and full virulence in a murine model, while CnGat1 is dispensable for proline and arginine utilization and survival in the host [27–29, 40, 41]. The *A. fumigatus* ortholog Af*areA* is required for virulence in a murine model of pulmonary aspergillosis [30].

The TOR pathway represents the main sensor for the intracellular nutrient state and mediates cellular responses, including alterations in the nitrogen and amino acid metabolism. The integration of extra- and intracellular nutrient-derived signals into other signaling and regulatory pathways is mediated via the nutrient-sensitive Tor kinase ScTor1 [36, 42]. Although

Table 1 Orthologs of the *S. cerevisiae* main regulatory systems for amino acid metabolism in *C. albicans*, *C. neoformans*, and *A. fumigatus* and their relevance for host-pathogen interaction

S. cerevisiae	*C. albicans*		*C. neoformans*		*A. fumigatus*	
	Orthologs	Phenotype	Orthologs	Phenotype	*Orthologs*	Phenotype
SPS system	CaSsy1 CaSsy5 CaPtr3 CaStp1 CaStp2	*stp2*Δ is attenuated in murine intravenous infection model [25]; *stp2*Δ and *ssy1*Δ are impaired in escape from macrophages [25, 26]; *ssy1*Δ, *ssy5*Δ, and *ptr3*Δ are slightly attenuated in murine intravenous infection model [26]	–	–	–	–
NCR	CaGat1 CaGIn3	Highly attenuated in murine intravenous infection model [27, 28]	CnGat1	Wild type phenotype in murine intranasal and *in vitro* macrophage infection model [29]	AfAreA	Attenuated in intranasal infection model in neutropenic mice [30]
TOR	CaTor1	–	CnTor1	Wild type phenotype in systemic murine and rabbit infection models [46]; Impaired TOR pathway results in attenuated virulence in murine intravenous infection model [31]	AfTor	–
GAAC	CaGcn4	Attenuated in murine intravenous infection model [32]	–	–	AfCpcA	Attenuated in murine pulmonary infection model [33]
ScGap1	CaGap1–6	–	CnAap1–8	*oap4*Δ/*oap5*Δ, *oap1*Δ/*aap2*Δ and *oap8*Δ strains are attenuated in *G. melonella* model, *aap4*Δ/*aap5*Δ in murine inhalation model [34, 35]	AfGap1	–

CaTor1 has not been studied in detail, it is assumed that it likewise influences the expression of NCR-associated genes and amino acid permeases (AAPs) [43], while Af*tor* was shown to be involved in amino acid metabolism [44]. The nitrogen or amino acid metabolism-related functions of CnTor1 have not been defined. The TOR pathway also influences fungal virulence, as demonstrated by its involvement in nutrient-dependent regulation of adhesins, hyphal morphogenesis, and chlamydospore formation in *C. albicans* [43, 45]. TOR signaling is also required for fluconazole tolerance and full virulence in a murine model in *C. neoformans* [31, 46]. Closely connected to TOR signaling is the general amino acid control (GAAC), which in *S. cerevisiae* induces amino acid biosynthesis upon starvation in a process that is mediated by the transcription factor ScGcn4 [36, 47]. Similarly, CaGcn4 is required for filamentous growth in response to amino acid starvation and for full virulence in systemic model of candidiasis [32, 48]. In *A. fumigatus*, *cpcA* encodes a functional ortholog of ScGcn4 [33]. To our knowledge, a functional ortholog of ScGcn4 is not described in *C. neoformans* and was also not identified via BLAST analysis performed within in the scope of this review (Table 1).

The Ssy1/Ptr3/Ssy5 (SPS) sensor system is the key element for monitoring extracellular amino acid concentrations in *S. cerevisiae*. Otherwise the organism is not really clear. [36, 49]. The system contains a functionally adapted, membrane-bound AAP named ScSsy1 which is activated upon increased extracellular concentrations of various amino acids, though the degree of activation varies by amino acid. SPS activation results in proteolytic cleavage of the inhibitory N-terminal domains of the cytosol retained transcription factors ScStp1 and ScStp2, which have redundant activity. Subsequently, ScStp1 and ScStp2 translocate into the nucleus and induce the expression of genes required for amino acid or protein utilization. The functionality of the system relies on the activity of ScShr3, an ER chaperone required for membrane localization of AAPs, including ScSsy1, which is therefore considered an upstream regulator of the SPS system [50, 51]. Like *S. cerevisiae*, *C. albicans* also senses extracellular amino acids via the SPS sensor system. Although CaSsy1 and ScSsy1 share a high degree of homology in the C-terminal domains, their N-terminal-sensing domains are highly divergent [52]. It is likely that this explains the altered CaSsy1 substrate specificity: the highest induction occurs in the presence of Arg, Asn, Asp, Gln, Glu, His, Lys, and Ser, while the other amino acids fail to stimulate the sensor [22, 52]. Further differences can be found in the downstream effectors CaStp1 and CaStp2 which, contrary to *S. cerevisiae*, exhibit a clear dichotomy in their regulatory activity: while the CaStp1 regulon contains genes required for protein utilization; like secreted aspartyl proteases (SAP) and oligopeptide transporters (OPT), CaStp2 is required for the expression of AAPs [53]. It could be hypothesized that the divergence is the result of the adaptation of *C. albicans* to the human host and is also relevant for virulence. Indeed, multiple components of the *C. albicans* SPS-sensing pathway have been implicated in virulence—for example CaSsy1 and CaStp2 are reported to be essential for macrophage phagosome alkalinization and immune cell evasion [25•, 26]. Additionally, deletion of Ca*CSH3*, the ortholog of Sc*SHR3*, resulted in impaired utilization of tryptophan and proline as nitrogen sources, filamentation, and reduced virulence in a murine model [54]. Ramachandra and colleagues have found several novel connections between *C. albicans* genes involved in the SPS-dependent amino acid sensing and NCR-mediated regulation, but the importance of such network interference and its impact on pathogenicity requires further characterization [28, 55].

To date, no functional SPS sensor systems have been described in *C. neoformans* or *A. fumigatus* nor could orthologs be found in their genomes via BLAST analysis conducted within the scope of this review, although it has been reported that *C. neoformans* possesses orthologs for ScSsy1 and ScStp2 [56•]. Putative orthologs of Sc*SHR3* are present in both *C. neoformans* and *A. fumigatus*, but have not been characterized. Therefore, it remains unclear how extracellular amino acids and oligopeptides are detected in these two organisms.

Another sensing mechanism for extracellular amino acids is provided by transceptors, which act simultaneously as transporters and receptors. ScGap1 is described as a broad-specificity transceptor for all L-amino acids, some D-amino acids, citrulline, and even some polyamines [57, 58]. ScGap1 undergoes a complex activation cycle involving internalization, ubiquitination, retention at vacuolar membranes, and subsequent re-recruitment to the cell membrane based on nitrogen source availability, and is tightly regulated by NCR [36, 59]. Additionally, ScGap1 acts as an inducer of the cAMP/PKA pathway in response to amino acid sensing [60, 61]. However, the precise mechanism by which ScGap1 activates downstream signaling pathways remains elusive [61, 62].

Initially, Ca*GAP1* was considered to be the primary, functional ortholog of Sc*GAP1* and linked to GlcNAc-induced hyphal morphogenesis via the cAMP/PKA pathway [63]. However, a recent study revealed six ScGap1 orthologs in *C. albicans*, with CaGap1, CaGap2, and CaGap6 also possessing transceptor activity, as demonstrated by their activation of PKA in complementation experiments performed in *S. cerevisiae* [64]. Interestingly, proof of cAMP/PKA induction via these orthologs in *C. albicans* could be not provided so far [65•]. Currently, it is unknown if *C. neoformans* and *A. fumigatus* have functional transceptors. *A. fumigatus* encodes for at least one uncharacterized Sc*GAP1* ortholog, while *C. neoformans* possesses a variety of amino acid permeases, but none seem to act as a transceptor [34, 56•].

Utilization and Uptake of Amino Acids and Oligopeptides in the Context of Pathogenesis

Fungi possess multiple secreted proteinases involved in nutrient utilization from host tissues. Secreted aspartic proteinases, in particular, foster the extracellular cleavage of proteins to oligopeptides and single amino acids, facilitating their uptake by fungi [66]. The SAP family in *C. albicans* encodes for 10 proteases and is well-studied regarding its impact on pathogenicity and fungal growth [67]. The hypothesis that SAPs liberate nutrients is supported by the fact that the eight *C. albicans* OPTs display differential expression in the presence of bovine serum albumin (BSA) [55, 68]. Indeed, mutant strains containing single knockouts for Ca*SAP1*, Ca*SAP2*, and Ca*SAP3* or a Ca*SAP4/5/6* triple knockout show impaired growth on BSA as the sole nitrogen source [69, 70]. Similarly, a triple knockout of Ca*OPT1/2/3* fails to grow on proteins as the sole nitrogen source [68].

Generally, proteinase activity also appears to be important during *C. neoformans* infection, since degradation of extracellular matrix and collagen has been observed in murine infection models and is viewed as a facilitator for establishing infections in the central nervous system via permeabilization of epithelial barriers [71–73]. Further, *C. neoformans* produces several secreted proteases involved in the utilization of immunoglobulin and complement proteins as the sole nitrogen and carbon source in vitro [74]. The aspartyl proteinase CnMay1 and the carboxypeptidase CnCxd1 have a broad specificity and are considered to supply *C. neoformans* with free amino acids liberated from extracellular protein sources and a strain lacking Cn*MAY1* was attenuated in a competition infection assay [75]. The functionality of oligopeptide transporters in *C. neoformans* virulence has not been investigated, although the fungal genome encodes for six predicted OPTs [76]. However, CnOpt1 was reported to be involved in a quorum-sensing process, thereby regulating the expression of virulence-associated genes [77].

A. fumigatus is a saprophytic organism that secretes a plethora of extracellular proteinases to provide nutrients for the cell. Its genome encodes for approx. 111 predicted proteases, which are at least partially induced upon various protein substrates and NCR-regulated via AfPrtT [78–83]. A mutant lacking Af*prtT* displayed markedly reduced expression of six secreted proteinases (Af*alp*, Af*mep*, Af*dpp4*, Af*cpdS*, AFUA_2G17330, and AFUA_7G06220) on protein substrates in vitro and reduced damage of lung epithelial cells and erythrocytes, but did not exhibit virulence defects in a murine model [81]. Generally, the role of proteinases in *A. fumigatus* virulence and nutrient acquisition is not well understood and difficult to investigate, due to their sheer number and functional redundancy. Nevertheless, the secretion of proteinases in vivo is shown to trigger host allergic responses [82, 84]. Interestingly, a mutant strain lacking all eight predicted members of the *OPT* gene family showed no growth defect on various nitrogen sources or virulence attenuation in a murine model of pulmonary aspergillosis [83]. This suggests either alternative uptake mechanisms or very efficient cleavage of host proteins to single amino acids.

In fungi, transport of amino acids into the cell occurs via AAPs with various efficiency and substrate specificity. The *C. albicans* genome contains at least 28 open reading frames (ORFs) encoding for AAPs [65•]. From this family, CaGap1–6 have been comprehensively studied [64, 65]. CaGap2 was identified as the functional ortholog of the general amino acid permease ScGap1, since it promoted growth on all tested amino acids as the sole source of nitrogen in a *S. cerevisiae* complementation assay [64]. This function as a general amino acid permease was further verified in *C. albicans*, where a strain lacking Ca*GAP2* failed to grow on Phe, Met, Val, Leu, Tyr, Trp, and Lys as the sole nitrogen source [65•]. CaGap6 also displayed a broad specificity, while CaGap1 and CaGap4 utilized only specific amino acids [64]. Interestingly, CaGap3 and CaGap5 were dispensable for growth on amino acids as a nitrogen source, yet their expression was nitrogen source-dependent, suggesting that they may complement the activity of the broad specificity permeases in as of yet unidentified host niches or conditions. The prominent role of CaGap2 is also reflected in its regulatory pattern: upon a shift from nitrogen starvation to nitrogen replete conditions, CaGap2 was downregulated at the transcript level and rapidly internalized, while the other *GAP*s were upregulated and the corresponding proteins remained on the plasma membrane (except for CaGap5). Interestingly, if this shift was reversed, only Ca*GAP2* and Ca*GAP6* were significantly induced [65•]. Moreover, the expression of all six Ca*GAPs* appears to be dependent on SPS activity in contrast to Sc*GAP1* [85, 86] (own unpublished data). Furthermore, at least CaGap2 is directly regulated by CaGat1 and CaGln3 [28].

Although various AAPs and transporters exist in *C. albicans* besides the GAP family, they remain poorly characterized. Based on predictions from *S. cerevisiae*, the *C. albicans* non-GAP permeases are considered to possess higher substrate specificity and possibly facilitate a rapid and precise adaptation to changing host environments in order to overcome the nutritional immunity induced by the host. An interesting example is Ca*PUT4*, an ortholog of the *S. cerevisiae* high-affinity proline permease gene Sc*PUT4*: although this permease is unstudied, it was shown that proline can serve simultaneously as the sole carbon and nitrogen source and its utilization is independent of NCR regulation [87•]. Further, proline sensing and uptake was independent of the SPS sensor system and the growth on proline as the sole nitrogen source was only slightly affected in a Ca*CSH3* knockout [53, 54].

The importance of proline in *C. albicans* pathogenesis was recently further underscored by the finding that proline catabolism-mediated cAMP/PKA activation induces hyphal morphogenesis [22•]. This study presents a new mechanism of amino acid–directed hyphal morphogenesis, where mitochondrial proline catabolism feeds the TCA cycle and leads to an accumulation of electron donors, which are subsequently used for the generation of ATP. The accumulated ATP then stimulates hyphal morphogenesis via the PKA pathway (Fig. 1) [22•]. It is further hypothesized that arginine uptake induces filamentation via the same pathway, since arginine is intracellularly converted to proline. Mitochondrial catabolism in some ways resembles a glucose-dependent process where high levels of glucose (2%) inhibit mitochondrial catabolism, while low concentrations (0.2%) activate oxidative respiration and stimulate proline- and arginine-induced filamentation [22•]. Nevertheless, filamentation is undoubtedly visible even in the presence of higher glucose concentrations, where

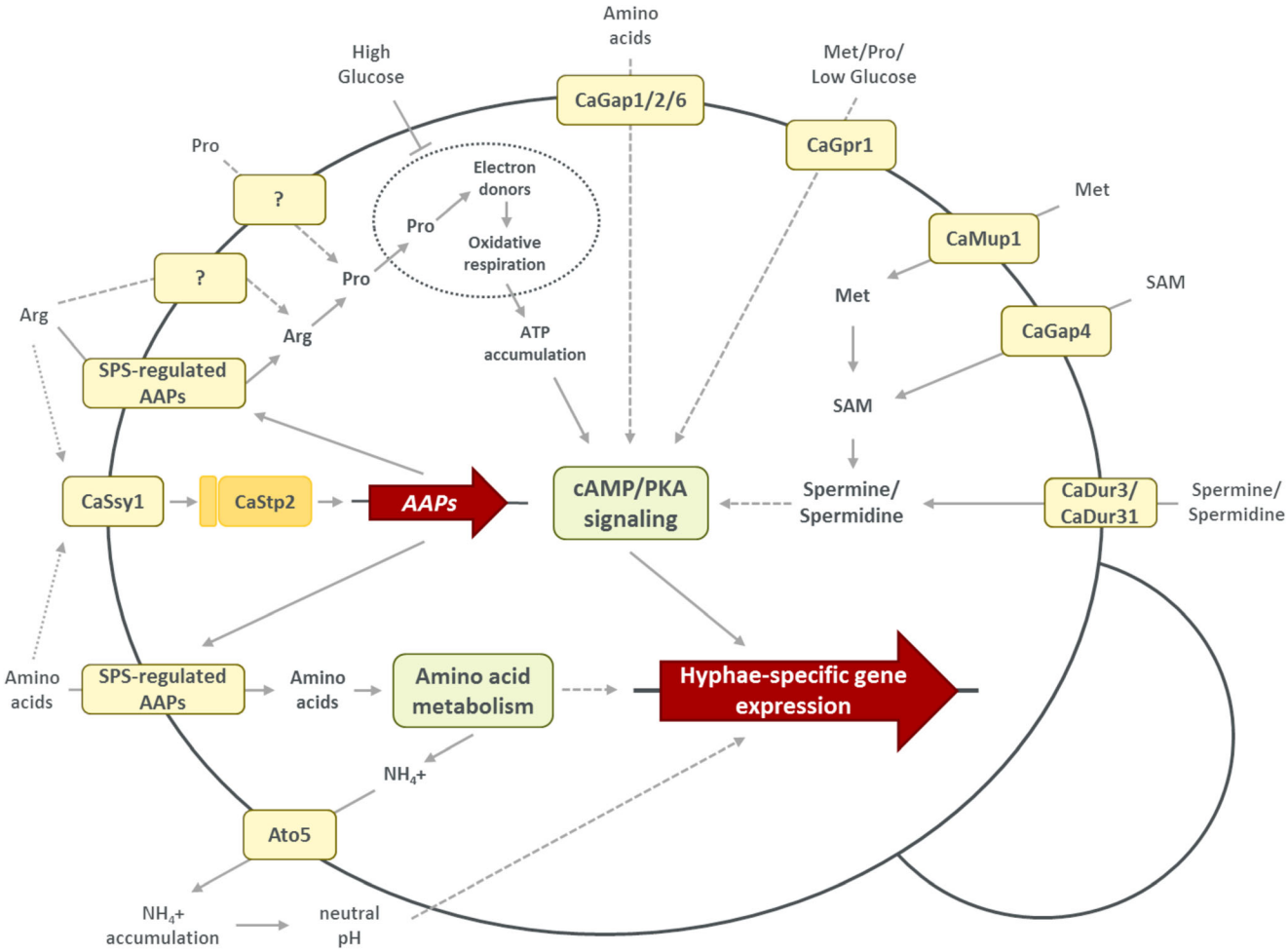

Fig. 1 Amino acid–induced hyphal morphogenesis in *C. albicans*. Given here is an overview of the described pathways for amino acid–induced hyphal morphogenesis in *C. albicans*. Known or suspected permeases and transporters are indicated with light yellow boxes, while the dark yellow box displays the transcription factor CaStp2. Verified mechanisms of amino acid sensing, transport, and regulation are indicated by solid arrows, while proposed, suspected or so far not completely resolved connections are indicated by dashed arrows. Dotted arrows show the sensory activity of CaSsy1. The dotted circle represents a mitochondrion. Illustrated is the activation of the cAMP/PKA-pathway and hyphal induction via the CaMup1-mediated uptake of methionine and its subsequent conversion via SAM to polyamines, like spermine and spermidine. The exact mechanism for this remains unresolved. A second hyphal inducer is provided via the mitochondrial degradation of proline, resulting in the generation of a high amount of electron donors, which are subsequently used for ATP generation via oxidative phosphorylation. The resulting accumulation of ATP also leads to an activation of the cAMP/PKA pathway. This process is inhibited by high amounts of extracellular glucose through the blocking of mitochondrial activity. The uptake of arginine and proline which drives this process is at least partially independent from the SPS sensor system and could involve unidentified permeases or transporters. Amino acid–driven alkalinization represents the third described hyphae-inducing pathway. Extracellular amino acids are sensed via CaSsy1, leading to CaStp2-mediated expression of amino acid permeases. The amino acids are taken up and metabolized, creating large amounts of cytotoxic ammonium, which are then extruded by ammonium transporters such as CaAto5 and lead to alkalinization of the environmental pH. This process results in hyphal morphogenesis, though the underlying mechanisms are not completely understood. CaGpr1 and CaGap1/2/6 are suspected to activate the cAMP/PKA pathway, although the nature of this activation and potential ligands have not been identified

mitochondrial activity should be suppressed [22•]. It is likely that in this case, filamentation is mediated via CaDur1, 2 activity, which hydrolyzes the urea by-product of the arginine to proline conversion into ammonia and carbon dioxide. This process is reportedly also involved in hyphal morphogenesis and considered a slower, alternate way for arginine-induced filamentation [19, 22•].

Interesting in this context is a previous study, which linked the G protein–coupled receptor CaGpr1 to proline and methionine-induced hyphal morphogenesis dependent on the quality and quantity of the present carbon source [88, 89] (Fig. 1). In *S. cerevisiae*, ScGpr1 physically interacts with glucose, sucrose, and mannose, activating of the cAMP/PKA pathway [90, 91]. In contrast, CaGpr1 appears dispensable for glucose-induced cAMP increase, though low glucose levels seem to be a prerequisite for the CaGpr1 methionine interaction [88, 89]. It could be speculated that the CaGpr1-mediated sensing of proline is necessary for the described hyphal morphogenesis, though it was shown to be SPS independent [22•]. However, the ligand(s) of CaGpr1 are not unequivocally identified and further research is required to elucidate CaGpr1-dependent signaling and its possible implications in arginine- and proline-induced filamentation [22•].

The CnGpr4 receptor in *C. neoformans* shows an activation pattern similar to CaGpr1, where it is internalized upon methionine exposure and required for methionine-induced elevation of cAMP, but is also dispensable for glucose-induced elevation. Nevertheless, a knockout mutant showed no attenuated virulence in a murine model [92]. Proline catabolism is also linked to *C. neoformans* virulence, since disruption of the catabolic pathway increased *C. neoformans* sensitivity to oxidative and nitrosative stresses, while a strain defective in proline catabolism had a mild defect in melanization and was avirulent in a murine model of intranasal infection [23•]. Like *C. albicans*, *C. neoformans* is also able to utilize proline as either a nitrogen and carbon source [23•, 87•]. However, in contrast to *C. albicans*, the corresponding catabolic enzymes are suppressed by primary nitrogen sources, pointing to NCR-mediated regulation [23•].

Another mechanism for amino acid–induced filamentation in *C. albicans* is through intracellular methionine metabolism and the uptake of methionine from the host via CaMup1 [21•] (Fig. 1). Methionine is subsequently metabolized to polyamines, like spermine or spermidine, via decarboxylation of the intermediate S-adenosylmethionine (SAM). Ultimately, this leads to induction of the cAMP/PKA pathway and hyphal growth, but the mechanism of the polyamine-mediated PKA pathway induction is not fully understood. Further, spermine and spermidine can also drive hyphal morphogenesis independently of CaMup1, likely via uptake through the characterized polyamine transporters CaDur3 and CaDur31 [93]. Another study showed that this pathway could be also triggered directly by the uptake of SAM via CaGap4 [65•]. Deletion of Ca*MUP1* results in decreased virulence in a model of disseminated candidiasis, shortened hyphae, and reduced survival during confrontation with macrophages [21•]. Methionine's intertwined roles in cellular metabolism, signaling, and virulence underscores the importance of this amino acid for *C. albicans*.

The methionine permeases CnMup1 and CnMup3 of *C. neoformans* are also involved in virulence, since they are required for proper capsule formation [34]. In addition to methionine, Cn*MUP1* and Cn*MUP3* are induced by histidine and tryptophan and are NCR-regulated [34]. Aside from these two, the *C. neoformans* genome encodes for 8 additional amino acid permeases with broader specificity, named Cn*AAP1–8* [56•]. In the presence of amino acids as the sole nitrogen source, *CnAAP2/4/5/8* were highly induced. Yet, with the exception of Cn*AAP4*, their expression was repressed upon the addition of ammonium sulfate, pointing to NCR-mediated regulation [56]. CnAap4 and CnAap5 are highly similar and to some degree redundant, as single knockout mutants still showed full virulence in a murine model, while a double knockout was avirulent [34]. Further, the double mutant is impaired in growth on every amino acid as a nitrogen source at 37 °C [34]. It could be speculated that the presence of both permeases represents a gene duplication that facilitated adaptation to the environment/host, since only CnAap5 is regulated by NCR. Recently, it was reported that the double knockout of Cn*AAP1* and Cn*AAP2* shows impaired growth on multiple amino acids as nitrogen sources and affects capsulation [35]. Interestingly, Cn*AAP1/2/4/5/6/8* are induced in the presence of the unfavorable carbon source, galactose [56]. It is therefore tempting to speculate that this represents a potential stimulus for utilization of amino acids as a carbon source in glucose-limited environments. Nevertheless, the regulatory pathways mediating amino acid sensing in vivo remain poorly understood and need to be investigated further.

For *A. fumigatus*, nearly nothing is known about the activity and role of AAPs. The genome encodes at least one Sc*GAP1* ortholog as well as multiple other predicted permeases. Transcriptome analyses suggest that amino acid uptake might play a role during infection, since amino acid metabolism is differentially regulated in confrontation to platelets in vitro and in whole blood [94, 95]. Furthermore, a predicted amino acid transporter was found to be upregulated in conidia confronted with human airway epithelia cells [96]. AAPs are also upregulated in the presence of proteins as a nitrogen source, suggesting the *A. fumigatus* degrades host proteins to scavenge for nutrients [83]. Indeed, genes involved in amino acid catabolism, including 13 AAPs and various secreted proteases, are induced during invasive aspergillosis, while amino acid biosynthetic pathways are downregulated [97]. It is hypothesized that this represents a clear starvation response, triggering the induction of genes regulating the utilization of alternative nutrient sources found in the lung. This is supported

by the concept that *A. fumigatus* relies heavily on the amino acids' valine, isoleucine, and methionine as carbon sources during invasive aspergillosis [98••]. This study proposes that the catabolism of these amino acids leads to the accumulation of propionyl-CoA, which is incorporated as pyruvate into the primary metabolism via the methylcitrate cycle-a pathway closely linked to the TCA cycle. Since disruption of methylcitrate synthase, a key element of the methylcitrate cycle, results in enhanced clearance by macrophages in vitro and strongly attenuated virulence in a murine model of invasive aspergillosis, it is hypothesized that acquisition of amino acids is important for *A. fumigatus* virulence [98••].

Amino Acid–Driven Alkalinization of the Environmental pH

The neutralization of the environmental pH, through the conversion of urea to carbon dioxide and ammonia by secreted ureases, is a well-known virulence factor in numerous plant and human pathogenic fungi [99]. For example, the ureolytic activity of *C. neoformans* modulates the pH of the macrophage phagolysosome, thereby affecting non-lytic exocytosis and intracellular replication of the yeast [100•]. In contrast, *C. albicans* does not encode a secreted urease. In this fungus, free ammonium is generated during arginine catabolism to ornithine and urea, and then subsequently processed via the intracellular enzyme urea amidolyase CaDur1, 2 to carbon dioxide and ammonia-both potent inducers of hyphal growth [101]. To balance out the potentially toxic quantity of ammonium ions in the cell, *C. albicans* has evolved a family of 10 ammonium transporters (ATOs), CaAto1–10 [102]. Although in a recent study, Danhof and Lorenz showed that deletion of Ca*ATO5* or the expression of a dominant-negative Ca$ATO1^{G53D}$ allele resulted in reduced ammonium release from the cell, the exact role of the ATOs is not understood [102]. Interestingly, despite the low enzymatic activity of CaDur1, 2, *C. albicans* alkalinizes the environmental pH with remarkable speed-in vitro growth on amino acids as the sole carbon source raises the environmental pH from 4.0 to neutral within 6–8 h [19]. This process is coordinated by CaStp2, which activation triggers the expression of most AAPs, OPTs, and ATOs along with amino acid metabolism genes, and results in amino acid uptake, ammonium extrusion, and hyphal morphogenesis [25•] (Fig. 1). Interestingly, the canonical regulator for pH adaptation and pH-induced hyphal morphogenesis, CaRim101, is dispensable for alkalinization [19], and alkalinization-driven hyphal morphogenesis (own unpublished data).

Environmental alkalinization can be observed upon in vitro growth in medium mimicking the nutrient composition of whole saliva and vaginal secretions in humans, suggesting that *C. albicans* could modulate the pH within the host [25•]. However, the contribution of this phenomenon to commensalism and/or pathogenicity in the oral cavity and vulvovaginal area remains unclear due to the lack of a suitable animal model that could represent the nutrient and pH conditions of the human host.

A few studies have demonstrated the importance of environmental neutralization in fungal escape from the macrophage phagosome. Indeed, the phagosome is hypothesized to resemble a glucose poor, but amino acid–enriched environment, which is evidenced by the fact that CaSsy1, CaStp2, and other proteins involved in the amino acid–driven alkalinization are activated upon phagocytosis and their absence renders the cell incapable of phagosomal escape due to failure to neutralize the phagosome [25•, 26, 102–104]. A recent study demonstrated that hyphal morphogenesis ruptures the phagosomal membrane, leading to luminal alkalinization, and contradicting the model of alkalinization as the cause of hyphal morphogenesis in the phagosome [105•]. Nevertheless, mutants defective in amino acid sensing also fail in filamentous growth, likely due to an inability to meet the growing energy needs of the cell following phagocytosis. The slower/impaired morphogenesis could allow the immune cell to repair the phagosomal membrane and prevent alkalinization. Further research is required to understand in greater detail the reason(s) for *C. albicans* hyphal morphogenesis within the macrophage phagosome.

Another observation worth discussing is that upon *C. albicans* phagocytosis, genes required for arginine biosynthesis are specifically upregulated [106]. This induction appears to be independent of CaGn4, a transcription factor that controls amino acid biosynthesis in response to starvation, but dependent on sublethal concentrations of reactive oxygen species (ROS) [48, 106]. Although the underlying mechanism behind this is not fully understood, it is evident that strains lacking key biosynthetic genes are defective in filamentation during phagocytosis [106].

Amino Acid Metabolism as a Potential Drug Target

Components of the amino acid metabolism represent an intriguing and novel target for antifungal therapy, since there are a variety of primary fungal metabolic pathways that are absent in the host [107]. This is particularly the case for the biosynthesis of 10 amino acids which are at least semi-essential for humans: Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Arg, and His. The potential of essential amino acid biosynthetic pathways as antifungal targets has been summarized previously [24, 108]. Another option would be targeting or exploiting fungal amino acid transport mechanisms. A current review of this strategy can be found here [109].

In contrast to what has been reported for *C. neoformans* and *A. fumigatus*, only a few reports exist concerning amino

acid biosynthetic pathways essential for virulence in *C. albicans* [24, 108]. For example, the disruption of the threonine and branched-chain amino acid biosynthetic pathways result in attenuated virulence in intravenous infection model in mice [110, 111]. It could be speculated that through *C. albicans'* adaptation to the human body as a commensal, it evolved sophisticated and specialized amino acid uptake systems to guarantee efficient supply and utilization within the host environment. Consequently, it could be more promising to target the responsible sensing and transport mechanisms in *C. albicans*, which lack human orthologs.

Conclusion and Future Perspectives

Amino acid utilization is essential for survival and infectivity of pathogenic fungi within the host. Yet, the factors and processes controlling amino acid–associated metabolism and virulence remain poorly understood to date. While the nature of amino acid uptake is partially described for *C. albicans* and *C. neoformans*, it is uncharacterized for *A. fumigatus*. Although the genome of the latter organism encodes for over a hundred secreted proteases, the functionality and specificity of only a few has been reported with no specific phenotypes, likely due to their redundant and overlapping functions. In addition, while strong evidence for the assimilation of carbon from valine, isoleucine, and methionine via the methylcitrate cycle during pulmonary aspergillosis exists, exactly how these extracellular amino acids are sensed and internalized remains elusive [98••]. Furthermore, the absence of orthologs for the SPS sensor system is striking and suggests either a more basal and nonspecific uptake mechanism, likely via a ScGap1 ortholog or a so far unidentified regulatory circuit.

Similar questions arise for *C. neoformans*, whose mechanisms for the sensing and uptake of extracellular amino acids have not yet been described. Further, the role of amino acid metabolism in infection remains unclear, though initial observations have been made [34]. Similarly, the role of amino acids in *C. albicans* morphogenesis, *A. fumigatus* melanization, and *C. neoformans* capsule formation remains to be investigated.

Due to the ability of *C. albicans* to assimilate different carbon sources simultaneously, it is tempting to speculate that a unique rewiring of NCR-mediated regulation of amino acid metabolism could provide similar advantages regarding nitrogen assimilation in this fungus [17]. First observations in this direction have already been made [87•], and a detailed investigation of these regulatory pathways could help in better understanding the nutritional basis of *C. albicans* colonization and virulence. A recent study showed differences in amino acid metabolism in *C. albicans* biofilms, with a strong upregulation of arginine, proline, aspartate, and glutamate metabolism in high biofilm-forming isolates, while low biofilm-forming isolates displayed an upregulation in purine, starch, and sucrose metabolism, suggesting that the ability to better metabolize amino acids leads to a growth advantage in nutrient-poor environments [20•]. However, the metabolic consequences arising from these differences and their outcome for the fungus, as well as the underlying regulatory systems, are unknown and require further investigations.

Altogether, amino acid metabolism drives growth and virulence of fungal pathogens within the host and further investigations are required to gain a more comprehensive understanding of the infection-relevant processes and their potential use as antifungal targets.

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Conflict of Interest Enrico Garbe and Slavena Vylkova each declare no potential conflicts of interest.

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- • Of importance
- •• Of major importance

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