The Involvement of the Mid1/Cch1/Yvc1 Calcium Channels in Aspergillus fumigatus Virulence

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Abstract

Aspergillus fumigatus is a major opportunistic pathogen and allergen of mammals. Calcium homeostasis and signaling is essential for numerous biological processes and also influences A. fumigatus pathogenicity. The present study characterized the function of the A. fumigatus homologues of three Saccharomyces cerevisiae calcium channels, voltage-gated Cch1, stretch-activated Mid1 and vacuolar Yvc1. The A. fumigatus calcium channels cchA, midA and yvcA were regulated at transcriptional level by increased calcium levels. The YvcA::GFP fusion protein localized to the vacuoles. Both ΔcchA and ΔmidA mutant strains showed reduced radial growth rate in nutrient-poor minimal media. Interestingly, this growth defect in the ΔcchA strain was rescued by the exogenous addition of CaCl2. The ΔcchA, ΔmidA, and ΔcchA ΔmidA strains were also sensitive to the oxidative stress inducer, paraquat. Restriction of external Ca²⁺ through the addition of the Ca²⁺-chelator EGTA impacted upon the growth of the ΔcchA and ΔmidA strains. All the A. fumigatus ΔcchA, ΔmidA, and ΔyvcA strains demonstrated attenuated virulence in a neutropenic murine model of invasive pulmonary aspergillosis. Infection with the parental strain resulted in a 100% mortality rate at 15 days post-infection, while the mortality rate of the ΔcchA, ΔmidA, and ΔyvcA strains after 15 days post-infection was only 25%. Collectively, this investigation strongly indicates that CchA, MidA, and YvcA play a role in A. fumigatus calcium homeostasis and virulence.

Introduction

Calcium ions are universally important secondary messengers involved in numerous signaling pathways in all eukaryotic cells. Calcium-mediated signaling regulates a diverse array of biological processes including gene transcription, protein conformation, energy metabolism, endo- and exo-cytosis, cytokinetic arrangement, and cell physiology [1,2]. Additionally, in yeasts and filamentous fungi, Ca²⁺ is important for the regulation of cell cycle, sporulation, hyphal morphogenesis, hyphal orientation and pathogenesis [3–8]. Calcium homeostasis and signaling are based on the equilibrium between the accumulation and release of calcium from intracellular stores, such as the vacuole, endoplasmic reticulum (ER), mitochondria and golgi apparatus. In Saccharomyces cerevisiae free cytosolic Ca²⁺ concentrations are very low, but in response to external stimuli or stress, Ca²⁺ channels are opened and Ca²⁺ is rapidly mobilized from intracellular stores. Cytosolic Ca²⁺ binds to calmodulin that activates the phosphatase calcineurin, which in turn dephosphorylates the Crz1 transcription factor, resulting in its nuclear translocation, the modulation of gene transcription including calcium transporters, and the restoration of calcium homeostasis [9–12].

Calcium homeostasis is maintained by calcium channels, pumps and transporters. Calcium channels allow the passive flow of Ca²⁺ across cell membranes into the cytosol. Two major calcium uptake pathways including the high-affinity (HACS) and low-affinity (LACS) calcium uptake system have been identified in fungi [7], [8], [13–16]. HACS consists of the voltage-gated Ca²⁺ channel Cch1 [17], [18], the stretch-activated calcium channel/regulatory protein Mid1 [19] and the PMP22_Claudin superfamily member Ecm7 [14,20]. Voltage-gated calcium channels sense the membrane potential and upon its depolarization open a gate allowing Ca²⁺ influx [1]. Stretch-activated channels are activated by cell deformations associated with contact sensing and thigmotropism [1]. HACS is the major route of calcium entry into the cell when availability is low [7], [8], [14]. Alternatively, LACS is active when calcium availability is high and consists of the Fig. 1, a pheromone-inducible plasma membrane protein [7,21].
In *S. cerevisiae*, endoplasmic reticulum and environmental stresses as well as exposure to mating pheromone trigger a response of the HACS [7,19], [22–24]. In filamentous fungi, HACS mutants demonstrate impaired calcium metabolism and reduced vegetative growth. In *A. nidulans*, CchA and MidA are important for the regulation of conidiation, hyphal polarity and cell wall components in low-calcium environments [8]. In phytopathogenic fungi, such as *Magnaporthe oryzae* and *Gibberella zeae*, Mid1 and Cch1 mutants have minor defects in growth and development, but they are still virulent [25–27]. However, the deletion of *mid1* in the *Claviceps purpurea* resulted in a complete loss of plant virulence [28]. Recent studies have indicated that in the human fungal pathogens *Candida albicans* and *Cryptococcus neoformans*, HACS was essential for sensing, and adapting to, the human host [8], [29–31]. These studies showed that HACS was required for the response to various stresses and also the tolerance of antifungal compounds, suggesting that Cch1 and Mid1 calcium channels are important in virulence and could represent targets for novel antifungal compounds.

The fungal vacuole is a major store for calcium. Fungal vacuolar calcium transporters, such as Ca\(^{2+}\) ATPases and Ca\(^{2+}/H^+\) exchangers, are involved in removing Ca\(^{2+}\) ions from the cytosol and loading them into internal stores to avoid calcium toxicity [32]. In *S. cerevisiae*, *PMC1* is responsible for this process preventing growth inhibition by the activation of calciuretin in the presence of elevated calcium concentrations [33]. Conversely, a vacuolar membrane localized calcium channel, *YVC1*, which is a member of the transient receptor potential (TRP) family [34], [35] mediates calcium release from the vacuole in response to hypertonic shock [36], [37]. The null mutant of the *C. albicans* *YFC1* homologue showed a decreased stress response, altered morphogenesis and attenuated virulence [38], [39], suggesting that *yvc1* homologues may also play a role in pathogenicity. To date no functional analyses of *YVC1* homologues in filamentous fungi have been reported.

*Aspergillus fumigatus* is a major opportunistic pathogen and allergen of mammals [40], [41]. Multiple components of the calcium signaling pathway impact upon *A. fumigatus* virulence [6], [42–45]. In addition, three *PMCI* calcium transport homologues in *A. fumigatus*, *pmcA-C*, were shown to be involved in calcium and manganese metabolism, but only *pmcA* influenced virulence [45]. Subsequently, the present study characterized the *A. fumigatus* homologues of the *CCH1*, *MID1* and *YVC1* calcium channels. The phenotypes of the constructed cca, mida and yvcA null mutants demonstrated their involvement in calcium metabolism and homeostasis. The *A. fumigatus* cca, mida, and yvcA null mutants were avirulent in a murine model of invasive pulmonary aspergillosis. Therefore, HACS and the release of vacuolar calcium stores are essential for full virulence.

**Results**

Identification of *A. fumigatus* Mid1, Cch1 and Yvc1 homologues

In the *A. fumigatus* genome a single gene showed significant identity to each of the *S. cerevisiae* calcium channel encoding genes, *CCH1*, *MID1* and *YVC1*. These *A. fumigatus* gene were subsequently named *cca* (*Afu1g11110*), *midA* (*Afu5g05840*) and *yvcA* (*Afu3g13490*). The predicted 2,125 amino acid sequence of *cca* encoded four hydrophobic transmembrane domains (Figure 1A). The 642 amino acid protein of *midA* contained an N-terminal signal peptide and six putative transmembrane domains

![Figure 1. The identification of CchA, MidA, and YvcA orthologues in A. fumigatus.](http://www.plosone.org/)

**Figure 1.** The identification of CchA, MidA, and YvcA orthologues in *A. fumigatus*. (A) CchA, (B) MidA, and (C) YvcA. ion_trans 1 to 4 indicate four domains Pf00520 and TMPred program. Each with six transmembrane-spanning regions. HR and SP indicate a hydrophobic regions and a signal peptide, respectively. The topologies were predicted using SMART database and TMpred program. The signal peptide was predicted by using SignalP. doi:10.1371/journal.pone.0103957.g001

**Figure 2.** mRNA accumulation of the *A. fumigatus* cca, midA, and yvcA genes. The wild-type and mutant strains were grown for 16 hours in YG medium at 37°C and the mycelia was either transferred to YG without any calcium for 30 minutes (Control:C) or for YG supplemented with 200 mM of calcium. RT-qPCR was used to quantify calcium channel mRNA abundance, which was normalized using *b-tubulin* (*Afu1g10910*). The relative quantitation of cca, midA, yvcA and *b-tubulin* was determined by comparison with a standard curve. (Ct – values plotted against logarithm of the DNA copy number). The results presented are the means (± standard deviation) of four sets of experiments. doi:10.1371/journal.pone.0103957.g002
The 670 amino acid protein encoded by *yvcA* had seven putative transmembrane domains (Figure 1C). The overall topology of the three calcium channels in *A. fumigatus* was similar to that of Ca$^{2+}$ voltage-gated channels in higher eukaryotes. The *A. fumigatus* midA, *cchA*, and *yvcA* are single copy genes and do not have paralogues (Figure S1 and Table S1). Moreover, they have very close homologues in other ascomycetes but more distantly related ones in *S. pombe*, *C. albicans*, and basidiomycetes (Figure S1).

To determine if the predicted calcium channels in *A. fumigatus* were influenced at the transcriptional level by calcium, *cchA*, *midA*, and *yvcA* mRNA accumulation was assessed in the parental strain when exposed to 200 mM CaCl$_2$. Calcium exposure for 10 and 30 minutes increased *yvcA*, *cchA*, and *midA* mRNA abundance by 3- and 9-fold, none and 22-fold, and 12- and 5-fold, respectively (Figure 2). These results suggest that *cchA*, *midA* and *yvcA* are regulated at transcriptional level by calcium. YvcA localization was assessed using a C-terminal GFP fusion protein. Growth of the YvcA::GFP strain resembled the parental strain under nutrient-rich and nutrient-poor conditions (data not shown), suggesting that the replacement of the wild-type gene with the GFP fusion construct in the transformed strain did not impair YvcA function. Within the hyphae the YvcA fusion protein localised to structures that resemble vacuoles during growth on minimal or complete media (Figure 3). The vacuolar identity of these structures was confirmed by staining the germings with the vacuolar specific fluorophore CMCA (Figure 3, see insets). We were not able to see any increase or relocalization of the YvcA::GFP upon exposure to 200 mM of CaCl$_2$ for 10 to 60 minutes (data not shown). These results strongly indicate YvcA is located on the vacuoles.

**Construction of the *A. fumigatus* cchA, midA and yvcA null mutants**

The function of *cchA*, *midA* and *yvcA* was investigated by generating the *ΔcchA*, *ΔmidA*, and *ΔyvcA* strains using a gene replacement strategy (Figure S2). In addition, a *ΔcchA ΔmidA* double mutant was created by inserting a deletion *midA::pyrG* cassette into *ΔcchA pyrG* mutant strain aiming to assess possible unique functions of each gene. The individual gene deletions were also complemented with the corresponding wild-type genes aiming to confirm the occurrence of possible secondary mutations during the construction of the deletion strains. The *ΔcchA*, *ΔmidA* and *ΔcchA ΔmidA* strains demonstrated reduced radial growth on solid MM, when compared to the parental strain (Figure 4A). Interestingly, the addition of different concentrations of CaCl$_2$ rescued the growth defect of the *ΔcchA* (25 to 200 mM), *ΔmidA*
(25 and 50 mM), and \textit{AcchA} \textit{AmidA} (25 and 50 mM) (Figure 4A). This positive CaCl₂ effect reflects a compensation of lacking the calcium channels for suitable levels of intracellular calcium. All single, or double, gene deletion mutants and the respective complemented strains displayed parental-like responses to temperature, cell wall (congo red and calcofluor white), membrane (SDS), oxidative (t-butyl) and osmotic stress (NaCl and Sorbitol). However, the \textit{DcchA}, \textit{DmidA} and \textit{DcchA DmidA} strains demonstrated increased sensitivity to the oxidative stress inducer, paraquat (Figure 4A). The \textit{DyvcA} mutant showed no differential sensitivity to any of agents tested (data not shown). Curiously, no growth reduction was observed in liquid MM and YG media (Figure 4B). None of the three single gene deletions had an impact upon conidiation or germination rate (data not shown). The complemented \textit{DcchA::cchA⁺}, \textit{AmidA::midA⁺} and \textit{DyvcA::yvcA⁺} showed parental phenotypes, indicating that the observed phenotypes were due to the gene deletion(s) in the respective strains (Figure 4A).

Cyclosporine A (CsA) is an immunosuppressive drug that inhibits calcineurin signaling by forming a complex with the immunophilin, cyclophilin, which then inhibits calcineurin. The \textit{AcchA}, \textit{AmidA} and \textit{AcchA AmidA} strains showed an increased resistance to cyclosporine in both MM and YAG; the \textit{DyvcA} mutant was as sensitive as the wild-type strain (Figure 5). Growth in the presence of the Ca²⁺-chelating agent EGTA was subsequently utilized to assess the impact of the gene deletions during calcium limitation. Growth of the parental strain was not dramatically affected by the presence of 10 or 20 mM EGTA, while \textit{AcchA} growth was restricted at 10 or 20 mM and \textit{AmidA} growth was restricted at only 20 mM (Figure 6). As previously, the \textit{AcchA AmidA} demonstrated a phenotype reminiscent of the \textit{AmidA}. Interestingly, the presence of EGTA resulted in the \textit{AcchA, AmidA, and AcchA AmidA} strains resembling the \textit{A. fumigatus} calcineurin catalytic subunit deletion [6], i.e., thicker swollen hyphae with dichotomous hyphal tips (Figure 6). Similar to the parental strain, EGTA had limited influence on the growth of the \textit{DyvcA} mutant (Figure 6).

Calcium is an important cellular signal for fungal drug resistance [46]. The \textit{AcchA} and \textit{AmidA} strains were more susceptible to various fungicides including voriconazole, ketoconazole, and itraconazole, while \textit{AcchA} was also more susceptible to posaconazole (Figure 7). In contrast, both the \textit{AcchA} and \textit{AmidA} strains were more resistant to caspofungin (Figure 7). The \textit{DyvcA}
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Figure 6. Restriction of external Ca$^{2+}$ supply by the addition of Ca$^{2+}$-chelating agent EGTA affects the growth of the ΔcchA and ΔmidA mutant strains. The parental, ΔcchA, ΔmidA, and ΔcchAΔmidA strains were grown in liquid MM supplemented with 0, 10 or 20 mM for 16 hours at 30°C. Bars, 5 μm.
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mutant showed no difference in susceptibility, from the parental strain, to any of the antifungal agents tested (data not shown).

The concentration of free calcium within the cell was evaluated in the A. fumigatus parental, ΔcchA, ΔmidA, and ΔyvcA mutant strains using Fluo-3 (Invitrogen), which is a highly sensitive fluorescent dye for the rapid measurement of calcium. Fluo-3 passively diffuses across cell membranes. Within the cell, the esters are cleaved by intracellular esterases yielding a fluorescent indicator that can no longer diffuse across membrane. Upon Ca$^{2+}$-binding, Fluo-3 exhibits an absorption shift from 506 to 526 nm. Thus, the relative intracellular Ca$^{2+}$ concentration was evaluated based upon the fluorescence ratio after dual-wavelength excitation. During the exposure to extracellular calcium concentrations from 0, 100, and 200 mM, in the parental strain there was an increase in intracellular calcium (Figure 6). The ΔyvcA, ΔcchA and ΔmidA strains showed a constant low level of intracellular calcium. Alternatively, the ΔcchA strain demonstrated higher intracellular calcium than the parental strain when in the presence of 0, 20, and 100 mM extracellular calcium (Figure 8). Collectively, these results suggest that CchA and MidA play an important role in the calcium uptake while YvcA is important for removing high calcium concentrations from the cytoplasm.

Recently, C. albicans ability to adhere to a polystyrene surface and buccal epithelial cells was inhibited by exposure to verapamil, a calcium channel blocker [47]. Biofilm formation on polystyrene was significantly inhibited about 25 to 30% in the ΔmidA and ΔyvcA mutant strains when measured by crystal violet staining (Figure S3). These results were further confirmed by a reduction of about 20% biomass in both strains when grown in static growth on polystyrene Petri dishes (Figure S3). As previously shown, all the mutant strains have growth comparable to the wild-type strain (Figures 4B and 8).

The A. fumigatus ΔcchA, ΔmidA and ΔyvcA mutant strains are avirulent in a low dose murine infection model

The involvement of CchA, MidA and YvcA in A. fumigatus pathogenicity was assessed in a neutropenic murine model of invasive pulmonary aspergillosis. Infection with the parental strain resulted in 100% mortality at 15 days post-infection, while ΔcchA, ΔmidA, and ΔyvcA infection resulted in a significant reduction ($p<0.005$) in mortality rate, with approximately 25% mortality after 15 days (Figures 9A, 10A and 11A). Full virulence was restored in independent strains resulting from the single ectopic reintegration of the parental cchA, midA, and yvcA gene (Figure S2) and the complemented strains (Figures 9A, 10A, and 11A), directly linking the attenuation of virulence to the disruption of three calcium channels.

Histopathological examinations of the parental, ΔcchA, ΔmidA and ΔyvcA infected tissues were performed to identify the differences in growth rate, tissue invasion and the inflammatory response. After 72 hours post-infection the lungs of mice infected with the parental strain contained multiple sites of invasive hyphal growth that penetrated the pulmonary epithelium in major airways (Figures 9B, 10B, and 11B) and formed pockets of branched invading hyphae originating from the alveoli (Figure 9B, 10B, and 11B). In contrast, ΔcchA, ΔmidA, and ΔyvcA infections typically contained inflammatory infiltrates in bronchiolies, some of which contained poorly germinated or ungerminated conidia (Figures 9B, 10B, and 11B). Fungal burden measured by real-time PCR showed that the growth of the ΔcchA, ΔmidA and ΔyvcA strains within the lungs was less than the parental and complemented strains (Figures 9C, 10C, and 11C, $p<0.0001$). This data strongly indicates that CchA, MidA, and YvcA play a role in A. fumigatus virulence.

Discussion

Calcium homeostasis and signaling is essential for numerous biological processes and importantly impacts upon the growth, stress tolerance and virulence of fungal pathogens [1], [48], [49]. Several elements of the calcium signal transduction machinery sense and transport Ca$^{2+}$, which in turn translate the spatiotemporal fluctuation in Ca$^{2+}$ levels into a cellular response [48]. The filamentous ascomycete A. fumigatus is an opportunistic pathogen and major allergen of mammals [40,41]. Multiple components of the calcium signaling cascade have previously been shown to impact upon A. fumigatus calcium homeostasis and virulence...
For example, the calcineurin catalytic subunit \( \text{calA} \) deficient strain had severe defects in hyphal extension, branching and conidial architecture [6], while the downstream calcineurin-activated transcription factor \( \text{CrzA} \) directly controlled the transcription of calcium transporters \( \text{pmcA-C} \) [45]. Significantly, all the \( \text{A. fumigatus} \) \( \text{DcalA} \), \( \text{DcrzA} \), and \( \text{DpmcA} \) mutants were avirulent in the murine model of invasive pulmonary aspergillosis [6,42,45].

Generally, cytosolic \( \text{Ca}^{2+} \) concentrations are very low (50 to 100 nM). Calcium channels, pumps, and transporters are essential to control intracellular calcium levels. However, how \( \text{A. fumigatus} \) uptakes and stores calcium is unclear. \( \text{Ca}^{2+} \) influx and efflux channels such as \( \text{Mid1}, \text{Cch1}, \text{and Yvc1} \) from \( \text{S. cerevisiae} \) have been identified in several filamentous fungi and linked to pathogenesis [54], [55]. Therefore, the function of three calcium channels in maintaining \( \text{Ca}^{2+} \) homeostasis during \( \text{A. fumigatus} \) infection was investigated. Growth in the presence of calcium-chelator \( \text{EGTA} \) represents calcium limiting conditions that would require \( \text{HACS} \). The \( \text{AchA} \) and \( \text{AmidA} \) strains demonstrated growth defects reminiscent of \( \text{AcalA} \) during growth under such conditions, strongly indicating that \( \text{A. fumigatus} \) \( \text{MidA} \) and \( \text{CchA} \) are important for calcium uptake in low-calcium environments. Moreover, the accumulation of intracellular calcium was lower in the \( \text{DmidA} \) and \( \text{AchA} \) strains, while the fact that \( \text{AchA} \) and \( \text{AmidA} \) were also more resistant to cyclosporine, suggests a low level of calcineurin activity and implies that intracellular calcium level were low. The exogenous supply of high extracellular calcium concentrations restored the growth defect of \( \text{AchA} \) and \( \text{AmidA} \) strain.

In \( \text{S. cerevisiae} \), \( \text{Mid1} \) is located in the plasma membrane as well as in the ER membrane [56], while in \( \text{Botrytis cinerea} \) the \( \text{Mid1} \) homologue is located to the ER membrane and nuclear envelope [16]. The interaction between \( \text{Mid1} \) and \( \text{Cch1} \) has been demonstrated in \( \text{S. cerevisiae} \) by co-immunoprecipitation [57], [58] and in \( \text{A. nidulans} \) using the yeast two-hybrid system [8]. However, in \( \text{B. cinerea} \) a split ubiquitin-based yeast two-hybrid approach did not reveal any interaction between \( \text{Mid1} \) and \( \text{Cch1} \) [16]. Although \( \text{Cch1} \) and \( \text{Mid1} \) have been shown to physically interact in various fungal systems, it remains to be addressed if \( \text{A. fumigatus} \) strains have a similar interaction.

**Figure 7. Susceptibility of \( \text{A. fumigatus} \) parental and calcium channel mutant strains to antifungal agents.** YG agar plates were inoculated with \( 2 \times 10^7 \) conidia. Etest strips for voriconazole, amphotericin, Anidulafungin, ketoconazole, itraconazole, and posaconazole were overlaid on the media, and the plates were incubated at 37°C for 16–20 hours. Representative pictures showing the inhibition ellipses and the endpoint values obtained with the Etest strips are shown. Experiments with all antifungal drugs were performed in triplicate.

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| Antifungal Agents | Wild-type | \( \Delta \text{cchA} \) | \( \Delta \text{midA} \) |
|-------------------|-----------|----------------|----------------|
| Voriconazole      | 0.094     | 0.064          | 0.064          |
| Amphotericin      | 0.75      | 0.75           | 0.75           |
| Anidulafungin     | 0.002     | 0.002          | 0.002          |
| Caspofungin       | 0.002     | 0.016          | 0.004          |
| Ketoconazole      | 32        | 6              | 8              |
| Itraconazole      | 2         | 0.5            | 1              |
| Posaconazole      | 0.125     | 0.094          | 0.125          |

[6,33,42], [49–53]. For example, the calcineurin catalytic subunit \( \text{calA} \) deficient strain had severe defects in hyphal extension, branching and conidial architecture [6], while the downstream calcineurin-activated transcription factor \( \text{CrzA} \) directly controlled the transcription of calcium transporters \( \text{pmcA-C} \) [45]. Significantly, all the \( \text{A. fumigatus} \) \( \text{AcalA}, \text{AcrzA}, \text{and ApmcA} \) mutants were avirulent in the murine model of invasive pulmonary aspergillosis [6,42,45]. Generally, cytosolic \( \text{Ca}^{2+} \) concentrations are very low (50 to 100 nM). Calcium channels, pumps, and transporters are essential to control intracellular calcium levels. However, how \( \text{A. fumigatus} \) uptakes and stores calcium is unclear. \( \text{Ca}^{2+} \) influx and efflux channels such as \( \text{Mid1}, \text{Cch1}, \text{and Yvc1} \) from \( \text{S. cerevisiae} \) have been identified in several filamentous fungi and linked to pathogenesis [54], [55]. Therefore, the function of three calcium channels in maintaining \( \text{Ca}^{2+} \) homeostasis during \( \text{A. fumigatus} \) infection was investigated. Growth in the presence of calcium-chelator \( \text{EGTA} \) represents calcium limiting conditions that would require \( \text{HACS} \). The \( \text{AchA} \) and \( \text{AmidA} \) strains demonstrated growth defects reminiscent of \( \text{AcalA} \) during growth under such conditions, strongly indicating that \( \text{A. fumigatus} \) \( \text{MidA} \) and \( \text{CchA} \) are important for calcium uptake in low-calcium environments. Moreover, the accumulation of intracellular calcium was lower in the \( \text{DmidA} \) and \( \text{AchA} \) strains, while the fact that \( \text{AchA} \) and \( \text{AmidA} \) were also more resistant to cyclosporine, suggests a low level of calcineurin activity and implies that intracellular calcium level were low. The exogenous supply of high extracellular calcium concentrations restored the growth defect of \( \text{AchA} \) and \( \text{AmidA} \) strain.

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Mammalian cells, Ca²⁺ response and hyphal development [20]. During the aging of role in HACS, which is also involved in the oxidative stress albicans homologue Ecm7 is an integral protein membrane with a Cch1p-Mid1p was essential to tolerate this condition. The iron, that iron stress led to increased oxidative stress and that relative Ca²⁺ strains were determined using the calcium-sensitive dye Fluo-3. The D intracellular calcium in the parental, S. cerevisiae [17,18,59], C. albicans This was observed in several species such as hypothesis of unique gene functions for either Cch1 or Mid1. complementing and contrasting phenotypes, resulting in the calcium channel mutants respectively were non-pathogenic or attenuated virulence in a murine model of pulmonary aspergillosis. A group of 10 mice per strain was intranasally infected with a 20 µl suspension of conidiospores at a dose of 5.0×10⁶. (B) Histological analysis of infected murine lung was performed 72 hours after infection with the wild-type strain and it reveals invasion of the murine lung epithelium. (C) Fungal burden was determined 48 hours post-infection by real-time qPCR based on 18S rRNA gene of A. fumigatus and an intronic region of the mouse GAPDH gene. Fungal and mouse DNA quantities were obtained from the Ct values from an appropriate standard curve. Fungal burden was determined through the ratio between ng of fungal DNA and mg of mouse DNA. The results are the means (± standard deviation) of five lungs for each treatment. Statistical analysis was performed by using t-test (*, p<0.01). doi:10.1371/journal.pone.0103957.g009

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Figure 9. A. fumigatus cchA contributes to virulence in neutropenic mice. (A) Comparative analysis of parental, ΔcchA, and ΔcchA::cchA strains in a neutropenic murine model of pulmonary aspergillosis. A group of 10 mice per strain was intranasally infected with a 20 µl suspension of conidiospores at a dose of 5.0×10⁶. (B) Histological analysis of infected murine lung was performed 72 hours after infection with the wild-type strain and it reveals invasion of the murine lung epithelium. (C) Fungal burden was determined 48 hours post-infection by real-time qPCR based on 18S rRNA gene of A. fumigatus and an intronic region of the mouse GAPDH gene. Fungal and mouse DNA quantities were obtained from the Ct values from an appropriate standard curve. Fungal burden was determined through the ratio between ng of fungal DNA and mg of mouse DNA. The results are the means (± standard deviation) of five lungs for each treatment. Statistical analysis was performed by using t-test (*, p<0.01). doi:10.1371/journal.pone.0103957.g009

death ahead of infection and may not rely on the HACS. Recently, Jiang et al. [63] characterized the A. fumigatus Mid1 homologue. The phenotypes shown for the afmid1 null mutant mostly agreed with what was observed in the presented study. However, Jiang and colleagues [63] showed that the deletion of afmid1, referred to as Δmid1Δ in the current investigation, led to hypervirulence in the immunosuppressed mice model. The contrasting results observed by these authors concerning afmid1 virulence could be due to the

Figure 10. A. fumigatus midA contributes to virulence in neutropenic mice. (A) Comparative analysis of parental, ΔmidA, and ΔmidA::midA⁺ strains in a neutropenic murine model of pulmonary aspergillosis. Experimental details are described in Figure 8A. (B) Histological analysis of infected murine lung was performed 72 hours after infection with the wild-type strain and it reveals invasion of the murine lung epithelium. (C) Fungal burden was determined 48 hours post-infection by real-time qPCR based on 18S rRNA gene of A. fumigatus and an intronic region of the mouse GAPDH gene. Experimental details are described in Figure 8C. doi:10.1371/journal.pone.0103957.g010
The A. fumigatus strains used in this study were CEA17 (pyrG−) and CEA17-90 (as the parental in all the experiments). The media used were: complete medium composed of 2% w/v glucose, 0.2% w/v yeast extract, trace elements (YAG) or YLU (YAG supplemented with 1.2 g of uracil and uridine) and minimal medium (MM) composed of 1% glucose or 2% glycerol, trace elements and nitrate salts [65], pH 6.5. Solid versions of the aforementioned media were prepared via the addition of 2% w/v agar. Strains were grown at 37°C.

Construction of the A. fumigatus mutants

The cassettes for gene replacement were constructed by in vivo recombination in S. cerevisiae as previously described by Colot et al. [66]. Approximately 1.5 kb from the 3'-untranslated region (UTR), and 3'-UTR flanking region of the targeted genes were selected for primer design. The primers 5F and 3R contained a short sequence homologous to the multiple cloning site (MCS) of the pRS416 plasmid. Both the 5- and 3'-UTR fragments were PCR-amplified from A. fumigatus genomic DNA (gDNA). The pyrG inserted into the gene replacement cassettes was amplified from pCDA21 plasmid and was used to generate a marker for prototrophy in the mutant strains. Each fragment along with the BamHI/EcoRI cut pRS416 plasmid were transformed into the S. cerevisiae strain SC94721 using the lithium acetate method [67]. The transformant DNA was extracted according to Goldman, et al. [68]. The cassette was PCR-amplified from the plasmids utilizing TaKaRa Ex Taq DNA Polymerase (Clontech Takara Bio) and used for A. fumigatus transformation. Southern blot analyses were used to confirm a single homologous integration event at the targeted A. fumigatus locus. The deleted mutant strains were complemented by co-transforming the respective gene utilizing TaKaRa Ex Taq DNA Polymerase (Clontech Takara Bio) and for A. fumigatus transformation. Southern blot analyses were used to confirm a single homologous integration event at the targeted A. fumigatus locus. The deleted mutant strains were complemented by co-transforming the respective gene plus a 1 kb flanking regions together with the pHA1′z vector [69] and selecting for hygromycin resistance on MM containing 150 mg/ml of hygromycin B.

The C-terminal Yvc::GFP fusion protein was created by cloning the yvc ORF (minus the stop codon) in frame with the green fluorescent protein (GFP) gene. The gfp and yvc sequences were separated by four additional codons that after translation produce a four amino acid linker (glycine-threonine-arginine-glycine) [70]. First, the yvc ORF plus 500 bp of the 5'-UTR were amplified from gDNA of the wild-type strain using the primers yvc pRS416 5Fw and yvc SPACER GFP Rv. The GFP ORF was amplified from the pCDA21 plasmid using the primers GFP pyrG Fw and pyrG Rv. The selective marker pyrG was PCR amplified from the pCDA21 plasmid using the primers GFP pyrG Fw and pyrG Rv. Approximately 600 bp of the 3'-UTR were amplified using the primers Afu yvc 3Fw and Afu yvc 3Rv primers. As describe previously, the S. cerevisiae in vivo recombination system was used for production of the transformation cassette. The PCR-amplified cassette was transformed into the A. fumigatus wild-type strain. The primers used above are described in Table S2.

Materials and Methods

Ethics statements

The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO in January 27, 1978 in its articles 8th and 14th. All protocols used in this study were approved by the local ethics committee for animal experiments from the Campus of Ribeirão Preto from Universidade de Sao Paulo (Permit Number: 08.1.1277.53.6; studies on the interaction of Aspergillus fumigatus with animals]. All animals were housed in groups of five within individually ventilated cages and were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation (Principios Éticos na Experimentação Animal - Colegio Brasileiro de Experimentação Animal, COBEA) and Guiding Principles for Research Involving Animals and Human Beings, American Physiological Society. All efforts were made to minimize suffering. Animals were clinically monitored at least twice daily and humanely sacrificed if moribund (defined by lethargy, dyspnea, hypothermia and weight loss). All stressed animals were sacrificed by cervical dislocation. File S1 reports all the ARRIVE checklist for reporting animal studies.
Phenotypic assays

The E-tests (Biomerieux) were performed according to the manufacturer’s instructions as follows, 1 × 10^7 conidia were inoculated into 20 ml YAG medium containing half the concentration of agar. E-tests strips were placed on the surface. Plates were incubated at 37°C for 24 h. The minimum inhibitory concentration was determined by analyzing the growth inhibition formed around the strip of voriconazole, amphotericin B, caspofungin, ketoconazole, itraconazole, posaconazole, and anidulafungin.

YvcA::GFP microscopy

YVC::GFP conidia were grown on coverslips in 4 ml of MM for 16 h at 30°C. After incubation, the coverslips with adherent germings were left untreated or treated with CaCl2 (200 mM) for 10, 30 and 60 min. CMAC (CellTracker Blue CMAC - Molecular Probes) staining of vacuoles was performed as described previously [71]. After growth, 10 μM CMAC dye was added to the cultures for 15 min, at 30°C. Subsequently, the coverslips were rinsed with PBS and mounted for examination. Slides were visualized on a Carl Zeiss Observer Z1 fluorescence microscope using the excitation wavelength of 450 to 490 nm, and emission wavelength of 500 to 550 nm. DIC (differential interference contrast) images and fluorescent images were captured with an AxioCam camera (Carl Zeiss) and processed using AxioVision software (version 4.8).

Maintenance of intracellular calcium homeostasis investigation

A fluorescent calcium indicator (Fluo-3, AM; Molecular Probes) was used to investigate the maintenance of intracellular calcium homeostasis. For this 1 × 10^7 conidia were added to 15 ml MM and incubated for 5 hours at 37°C. A minimum of 3 replicates was performed for each strain. After incubation, the medium was discarded by centrifugation and the samples were resuspended in 2 ml PBS. Then, 200 μl of this cell suspension were placed in a 96-well, black/clear, flat bottom microtitre plate (BD Falcon) and a solution of CaCl2 (20, 100, or 200 mM) containing 10 μM Fluo-3 was added. Plates were incubated for 10 min at 30°C. Control samples were incubated for 10 min with Fluo-3 without calcium treatment. The samples were washed again with PBS and fluorescence at 526 nm determined on a SpectraMax i3 spectrometer (Molecular Devices).

Crystal violet assay and biofilm formation

Crystal violet assays were used to assess biofilm formation as described previously by Mowat et al. [72]. A. fumigatus biofilms were formed in 96-well microtitre plates (Corning® Costar®). Biofilms were formed by inoculating 1 × 10^4 conidia/ml in MM containing 0.1% glucose. The plates were incubated at 37°C for 16 hours. Subsequently, the media was discarded and the biofilms were carefully washed three times with sterile PBS to remove non-adherent cells. The biofilms were then dried at 60°C for 10 min and 200 μl of 0.5% crystal violet (Sigma) solution added. The plate was then incubated at room temperature for 5 min and then extensively washed with distilled water until all unbound crystal violet was removed. The plate was dried again and then 200 μl ethanol was added to each well to elute the bound crystal violet. The ethanol was then transferred to a clean 96-well microtitre plate and the absorbance read at 570 nm using a SpectraMax i3 spectrometer (Molecular Devices). A minimum of 6 replicates was performed for each strain.

In addition, A. fumigatus biofilm biomass was determined as described by Shopova et al. [73]. Briefly, conidial suspensions of 1 × 10^7 to 1 × 10^8 conidia/per well were inoculated in HEPES-buffered RPMI 1640 with L-glutamine. This suspension was inoculated into pre-sterilized polystyrene petri dish (static growth) or glass flasks (planktonic growth) and grown for 96 hours at 37°C. Biomass was determined by dry weight.

RNA extraction and real-time PCR reactions

Mycelia were harvested by filtration, washed twice with H2O and immediately frozen in liquid nitrogen. For total RNA extractions, the mycelia were ground in liquid nitrogen with pestle and mortar. Total RNA was extracted with Trizol (Invitrogen, USA). The integrity of the RNA from each treatment was assessed using an Agilent 2100 Bioanalyzer. RNase-free DNase I treatment was carried out as previously described by Semighini et al. [74]. Twenty micrograms of total RNA was treated with DNase I, purified using the RNaseasy kit (Qiagen) and cDNA synthesized using the SuperScript III First Strand Synthesis system (Invitrogen) with oligo(dT) primers, according to the manufacturer’s instructions. All the qPCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and SYBR Green PCR Master Mix (Applied Biosystems, USA). The reactions and calculations were performed according to Semighini et al. [74]. The primers used are described in Table S2.

Murine model of pulmonary aspergillosis, lung histopathology and fungal burden

The murine model of pulmonary aspergillosis was performed according to Dinamarco, et al. [45]. Outbreed female mice (BALB/c strain; body weight, 20 to 22 g) were housed in vented cages containing 5 animals. Mice were immunosuppressed with cyclophosphamide, which was administered intraperitoneally (150 mg/kg of body weight) on days -4, -1 and 2 prior to and post infection. Hydrocortisoneacetate (200 mg/kg) was injected subcutaneously on day -3. Fresh A. fumigatus conidia were grown on YAG for 2 days prior to infection, then harvested in PBS and filtered through Miracloth (Calbiochem). Conidial suspensions were spun for 5 min at 3,000 × g, washed three times with PBS, counted using a hemocytometer, and resuspended at a concentration of 2.5 × 10^6 conidia/ml. The viability of the administered inocula were confirmed by incubating a serial dilution of the conidial suspension, on YAG media, at 37°C. Mice were anesthetized by halothane inhalation and infected by intranasal instillation of 5.0 × 10^5 conidia in 20 μl of PBS. As a negative control, a group of 5 mice received PBS only. Mice were weighed every 24 h from the day of infection and visually inspected twice daily. In the majority of cases, the endpoint for survival experimentation was identified when a 20% reduction in body weight was recorded, at which time the mice were sacrificed. The statistical significance of comparative survival values was calculated using log rank analysis and the Prism statistical package. Additionally, at 3 days post-infection, 2 mice per strain were sacrificed and the lungs were removed, fixed, and processed for histological analysis.

Lung histopathology and fungal burden

After sacrifice, the lungs were removed and fixed for 24 hours in 5.7% formaldehyde–PBS. Samples were washed several times in 70% alcohol before dehydration in a series of alcohol solutions of increasing concentrations. Finally, the samples were diafanized in xylol and embedded in paraffin. For each sample, sequential 5-μm-thick sections were collected on glass slides and stained with Gomori methenamine silver (GMS) or hematoxylin and cosin (HE) stain following standard protocols [40]. Briefly, sections were...
deparaffinized, oxidized with 4% chronic acid, stained with methanamine silver solution, and counterstained with picric acid. For HE staining, sections were deparaffinized and stained first with hematoxylin and then with eosin. All stained slides were immediately washed, preserved with mounting medium, and sealed with a coverslip. Microscopic analyses were done using an Axiosplan 2 imaging microscope (Carl Zeiss) at the stated magnifications under bright-field conditions.

To investigate fungal burden in murine lungs, mice were infected as described previously, but with a higher inoculum of 1 × 10^6 conidia/20 μl. The higher inoculum, in comparison to the survival experiments, was used to increase fungal DNA detection. Animals were sacrificed 72 h post-infection, and both lungs were harvested and immediately frozen in liquid nitrogen. Samples were homogenized by vortexing with glass beads for 10 min, and DNA was extracted and the subsequent use of the phenol-chloroform method. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Six-point standard curves were calculated using serial dilutions of gDNA from all the strains used and the uninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle (CT) values from a appropriate standard curve. Fungal burden was determined as the ratio between the fungal and mouse DNA.

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**Author Contributions**

Conceived and designed the experiments: GHG MHSG. Performed the experiments: PAC JC LKW VLPB LNZR. Analysed the data: PAC JC LKW VLPB LNZR. Contributed reagents/materials/analysis tools: PAC JC LKW VLPB LNZR. Contributed to the writing of the manuscript: GHG NAB.

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