Riboflavin and pantothenic acid biosynthesis are crucial for iron homeostasis and virulence in the pathogenic mold Aspergillus fumigatus

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ABSTRACT

Background: Aspergillus fumigatus is the most prevalent airborne fungal pathogen, causing invasive fungal infections mainly in immunosuppressed individuals. Death rates from invasive aspergillosis remain high because of limited treatment options and increasing antifungal resistance. The aim of this study was to identify key fungal-specific genes participating in vitamin B biosynthesis in A. fumigatus. Because these genes are absent in humans they can serve as possible novel targets for antifungal drug development.

Methods: By sequence homology we identified, deleted and analysed four key A. fumigatus genes (riboB, panA, pyroA, thiB) involved respectively in the biosynthesis of riboflavin (vitamin B2), pantothenic acid (vitamin B5), pyridoxine (vitamin B6) and thiamine (vitamin B1).

Results: Deletion of riboB, panA, pyroA or thiB resulted in respective vitamin auxotrophy. Lack of riboflavin and pantothenic acid biosynthesis perturbed many cellular processes including iron homeostasis. Virulence in murine pulmonary and systemic models of infection was severely attenuated following deletion of riboB and panA, strongly reduced after pyroA deletion and weakly attenuated after thiB deletion.

Conclusions: This study reveals the biosynthetic pathways of the vitamins riboflavin and pantothenic acid as attractive targets for novel antifungal therapy. Moreover, the virulence studies with auxotrophic mutants serve to identify the availability of nutrients to pathogens in host niches.

Abbreviations: BPS: bathophenanthrolinedisulfonate; BSA: bovine serum albumin; CFU: colony forming unit; -Fe: iron starvation; +Fe: iron sufficiency; hFe: high iron; NRPSs: nonribosomal peptide synthetases; PKSs: polyketide synthetases; wt: wild type

INTRODUCTION

Invasive fungal infections have increased dramatically in number over the last 40 years, largely because of the increase in the global number of immunocompromised patients. It is estimated that over 1.5 million people/year are infected worldwide by the major invasive fungal pathogens Candida albicans, Aspergillus fumigatus and Cryptococcus neoformans [1,2]. Despite modern antifungal treatments, mortality rates remain between 20 to 90%. Treatment efficacy has plateaued and fungal resistance is developing [3].

Development of new drugs is hampered by the fact that fungi are eukaryotes and share with humans many of the same metabolic pathways. Therefore, most existing antifungals are not sufficiently selective and have various side effects.

Research has therefore focused on identifying essential genes and pathways not shared with the infected host [4–7]. Using a combination of comparative genomics, transcriptomics and metabolic flux analysis, we recently identified 64 fungal-specific targets including metabolic enzymes participating in amino acid, lipid and vitamin biosynthesis [8]. Of these, 18 targets have already been validated in the literature, including enzymes participating in the biosynthesis of aromatic amino acids [9], lysine [10,11], histidine [12] and cysteine/methionine [13] and in the biosynthesis of lipids, including phospholipids [14], fatty acids [6] and oxylipins [15]. However, the assessment of fungal-specific targets in the vitamin biosynthesis pathways and their importance during infection has not been well established.

In this study, we analysed for the first time the vitamin B biosynthetic pathways for thiamine (B1),...
riboflavin (B2), pantothenic acid (B5) and pyridoxine (B6) in the important human pathogenic mold *A. fumigatus*. These fungal pathways, including the encoding genes are shown in Supplementary Figure 1. They are not found in humans, who need to obtain the required vitamin intake from their food. We deleted key fungal-specific genes in each pathway and phenotypically evaluated the resulting auxotrophic mutants in vitro and in vivo during infection. We show that riboflavin and pantothenic acid biosynthesis are important for iron homeostasis in vitro and for establishing a lethal infection in mice, suggesting strategies for their development as antifungal targets.

### Materials and methods

**Strains and growth conditions**

Fungal strains were generally cultured on/in *Aspergillus* minimal medium (AMM) containing 1% glucose as carbon source and 20 mM glutamine as nitrogen source or on complete medium containing 2 g/L peptone and 1 g/L yeast extract [16]. Alternatively, YAG medium consisting of 0.5% yeast extract, 1% glucose, 10 mM MgCl₂, trace elements and vitamins, served as nutrient source. Supplements are indicated in the respective experiments. Iron was omitted for iron-depleted conditions (-Fe) and 30 µM FeSO₄ was added into the media for iron-replete (+Fe) conditions. Vitamin stock solution included 2.5 µM pantothenic acid, 10 µM pyridoxine and 10 µM niacin. Blood agar medium contained 1.8% agar, 0.5% sodium chloride and 10% or 25% blood. A hypoxic chamber (C-Chamber and Pro-Ox, Pro-CO₂ controller; Biosherics) with the settings 1% O₂, 5% CO₂ and 94% N₂ was used for hypoxic conditions. For growth assays, 10⁴ conidia were point-inoculated on minimal medium agar plates and incubated for 48 h at 37°C. For inoculation of 100 ml liquid medium, 10⁶/ml conidia were used. The *akuA*-deficient derivative of ATCC46645 AfS77, termed wild-type (wt) here, served as the reference recipient [17]. Primers used in this study are listed in Supplementary Table 1. Generation of the *A. fumigatus* mutant strains and their verification by PCR and Southern analysis is outlined in detail in Supplementary Figure 2A-M. Strains used in this study are listed in Supplementary Table 2.

**Analysis of extra-and intracellular siderophores and biomass production**

For quantification of extra- or intracellular siderophores, culture supernatants or lyophilized mycelia were saturated with FeSO₄ and extracted as described previously [18]. To analyse biomass production, mycelia from liquid cultures were freeze-dried and weighed.

For the vitamin-shift experiment, fungal strains were inoculated in liquid minimal medium supplemented with vitamins (riboflavin 2 µM, pantothenic acid 2 µM, pyridoxine 0.1 µM and thiamine 0.1 µM) for 12 h (supplementation phase). Germlings were extensively washed with water before shifting to vitamin-starved minimal medium for 36 h (starvation phase). Finally, the cultures were either vitamin supplemented (resupplementation phase) or not supplemented (continued starvation phase) for another 12 h. Dry weight was determined from freeze-dried mycelia.

**Identification of *A. fumigatus* flavoproteins from the proteome**

For screening flavin-dependent *A. fumigatus* proteins, the *A. fumigatus* Af293 proteome data set (AspGD; [http://www.aspergillusgenome.org/download/domains/A_fumigatus_Af293/](http://www.aspergillusgenome.org/download/domains/A_fumigatus_Af293/)) from the *Aspergillus* database [19] was searched with the terms “flavo”, “flavin”, “FMN” and “FAD”. The resulting list was compared with *A. fumigatus* proteins identified in AspGD ([http://www.aspgd.org](http://www.aspgd.org)) with terms “flavo”, “flavin”, “FMN” and “FAD” [20].

| Pathway (number of proteins) | 
|-----------------------------|
| Metalloreduction, e.g. reductive iron assimilation [17] | 
| Light regulation/response [4] | 
| Ergosterol biosynthesis [6] | 
| Amino acid biosynthesis [3] | 
| Respiration/TCA cycle [1] | 
| β-Oxidation [4] | 
| Purine catabolism [1] | 
| Nitrate assimilation/NO detoxification [5] | 
| REDOX homeostasis [4] | 
| Pyridoxin biosynthesis [1] | 
| Pantothenic acid biosynthesis [4] | 
| Nicotinic acid biosynthesis [2] | 
| Coenzyme A biosynthesis [2] | 
| Siderophore biosynthesis [1] | 
| Sulfur assimilation/methionine biosynthesis [4] | 
| Iron-sulfur-cluster biosynthesis [2] | 
| Secondary metabolism [13] | 
| Ubiquinone biosynthesis [2] | 
| Glycerophospholipid metabolism [1] | 
| Protein modification: sumoylation, neddylation, ubiquitination [6] |
(300 mg/kg) 3 days prior to infection, on the day of infection, and 3, 7, and 11 days postinfection. Fungal strains were grown for 3 days at 37°C on MM agar with appropriate vitamin supplementation. Spores were collected in PBS with 0.2% Tween 20 and counted by a hemocytometer. For the ΔriboB strain, conidial viability and germination were not affected on MM supplemented with 2.5 µM riboflavin (Supplementary Figure 3). The mice were infected intranasally with 5 × 10⁶ dormant spores, suspended in 20 µl of PBS plus 0.2% Tween 20 (10 µl in each nostril). Neutropenic (ii) pulmonary and (iii) disseminated models; six-week-old female ICR mice were immunocompromised with cyclophosphamide (150 mg/kg in PBS) injected intraperitoneally at 3 days prior to and at 2 days postconidial infection. Cortisone acetate (150 mg/kg PBS with 0.1% Tween 80) was injected subcutaneously at 3 days prior to conidial infection. For pulmonary infection, mice were infected intranasally as described above. For disseminated infection, 5 × 10⁵ dormant spores were injected through the lateral tail vein. Survival was monitored for up to 21 days. For histopathology, mice were sacrificed two days after infection, their lungs were removed and stained with Grocott’s methenamine silver stain (GMS; fungal staining) and hematoxylin and eosin (H&E; tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the second day post-infection, their lungs were removed and homogenized, and the homogenates were plated on YAG medium supplemented with the appropriate vitamin for colony forming unit (CFU) enumeration. Experiments were ethically approved by the Ministry of Health (MOH) Animal Welfare Committee, Israel.

**Results**

**Generation of vitamin B auxotrophic mutant strains in A. fumigatus**

To analyse the role of defined vitamin B biosynthetic pathways of *A. fumigatus*, we first identified by bioinformatics analysis, genes encoding key enzymes of the respective pathways for the synthesis of riboflavin (*riboB/Afu1g13300*, encoding GTP cyclohydrolase II), pantothenic acid (*panA/Afu5g11040*, encoding pantoate-beta-alanine ligase), pyridoxine (*pyroA/Afu5g08090*, encoding pyridoxal 5-phosphate synthase) and thiamine (*thiB/Afu2g08970*, encoding thiamine-phosphate diphosphorylase and hydroxyethylthiazole kinase) (Supplementary Figure 1 and Supplementary Tables 3–6). These genes were deleted in the *A. fumigatus* *akuA::loxP* recipient strain derived from ATCC46645 (AfS77, termed wt here), largely lacking non-homologous recombination [21,22], by homologous recombination and hygromycin selection as described in the Materials and Methods (Supplementary Figure 2). To confirm gene deletion-specific phenotypes, mutant strains were complemented (*C* strains) by re-integration of functional gene copies. Notably, we also attempted to generate a riboflavin auxotroph by deletion of *Afu2g16360*, the ortholog of *Histoplasma capsulatum* *rib2*, which is essential for riboflavin biosynthesis in this dimorphic fungal species [23]. Surprisingly, deletion of *Afu2g16360/rib2* did not result in riboflavin auxotrophy in *A. fumigatus* (data not shown), most likely because of the presence of paralogous genes (Supplementary Figure 1A).

**Deletion of riboB, panA, pyroA and thiB leads to auxotrophy**

The growth of the mutant strains was compared to the wt and complemented strains by spotting 10⁴ conidia on solid minimal medium, complete medium or blood agar, containing different concentrations of vitamin as shown in Figure 1. No visible growth was seen in the mutant strains in the absence of their respective vitamin supplementation, confirming that the gene deletions resulted in auxotrophy. Radial growth of the ΔriboB and ΔpanA mutants was fully restored by addition of 2.5 µM riboflavin or pantothenic acid (Figure 1(a,b)), respectively, whereas growth of the ΔpyroA and ΔthiB strains necessitated addition of only 0.1 µM of their respective vitamin (Figure 1(c,d)). Complemented strains showed wild-type phenotypes in all assays performed, confirming the specificity of gene deletion phenotypes (Figure 1). Complete medium allowed full growth and sporulation of the ΔpanA, ΔpyroA and ΔthiB strains, whereas in ΔriboB conidiation was blocked (reflected by the white instead of greenish colonies), most likely because the amount of riboflavin in the medium, which derives from the yeast extract component in this medium, is not sufficient to support wt-like growth for ΔriboB.

In contrast to the ΔpyroA and ΔthiB strains, both ΔriboB and ΔpanA did not grow on blood agar without vitamin supplementation, indicating that blood riboflavin and pantothenic acid levels are too low to support their growth (Figure 1(a–d)). Interestingly, for growth of ΔpanA on blood agar, increased pantothenic acid supplementation (10 µM) was required. This effect is most likely because pantothenic acid is adsorbed by serum albumin in the blood. In agreement, 1% bovine serum albumin (BSA) blocked growth of ΔpanA in the presence of 1 µM pantothenic acid in minimal medium, which supports growth without BSA (Figure 1(b)).

In liquid minimal medium without vitamin, after 24 h at 37°C, ΔriboB and ΔpanA conidia remained compact,
whereas ΔpyroA and especially ΔthiB conidia underwent significant swelling, indicating partial early germination (Figure 2(a), insets, Figure 2(b)). Interestingly, in liquid minimal medium, vitamin supplementation restored mutant growth at concentrations far lower than those needed on minimal medium agar plates (Figure 2).

Riboflavin and its derivatives FAD and FMN play a crucial role in NO detoxification and sporulation

Riboflavin is the precursor of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and as such used by approximately 238 flavoproteins in *A. fumigatus* (Supplementary Table 7). Flavoproteins have central metabolic and regulatory roles; Table 1 displays 22 pathways comprising 91 of the 238 *A. fumigatus* flavoproteins. FAD is a crucial coenzyme of flavohemoglobins, which provide protection against NO and related reactive nitrogen species [24]. We therefore tested the growth of ΔriboB during NO stress, induced by addition of 5 mM NaN₃ at a low pH of 4.0 (acidic conditions stimulate generation of nitric oxide from nitrate [25]), and limiting 2.5 µM riboflavin for growth. Growth of the mutant was inhibited compared to the wt and complemented strains, indicating an important role for riboflavin in nitric oxide (NO) detoxification (Figure 3(a)). FAD is also a crucial coenzyme for the biosynthesis of the B vitamins niacin, pantothenic acid and pyridoxine. We therefore tested whether we can correct the impaired sporation of ΔriboB on minimal medium (reflected by a decrease in greenish pigmentation of the colony) in the presence of 2.5 µM riboflavin, by adding these vitamins on minimal medium. We found that supplementation of ΔriboB with 2.5 µM pantothenic acid, 10 µM pyridoxine and 10 µM niacin restored conidiation, suggesting a role of riboflavin in biosynthesis of these vitamins (Figure 3(b), Table 1).

The vitamins riboflavin, pantothenic acid, pyridoxine and thiamine have potent anti-oxidative activity. We therefore tested the sensitivity of the ΔriboB, ΔpanA, ΔpyroA and ΔthiB strains to survive oxidative stress induced by H₂O₂ or menadione under partial supplementation with their respective vitamin. However, we found no differences in the sensitivity of the mutants compared to the wt (data not shown).

Decreased riboflavin availability reduces production of siderophores in *A. fumigatus*

To survive in the human host under conditions of iron starvation, *A. fumigatus* employs two high-affinity iron-uptake systems, reductive iron assimilation and the extracellular siderophores fusarinine C (FsC) and triacetylfusarinine C (TAFC), as well as the intracellular siderophore ferricrocin (FC) for iron storage and distribution [26,27]. To further characterize the role of riboflavin biosynthesis in iron utilization and siderophore production, wt and ΔriboB were analysed after growth in liquid minimal medium during iron starvation (-Fe), iron sufficiency (+Fe, 30 µM FeSO₄) and iron excess.
(hFe, 5 mM FeSO₄) supplemented with either a low (0.1 µM) or a high (2.5 µM) riboflavin concentration (Figure 4). Biomass production was significantly decreased in ΔriboB compared to wt under low riboflavin and iron sufficiency (74% less, P < 0.0001 vs. wt) or iron excess (67% less, P < 0.0001 vs. wt) (Figure 4(a)). Siderophore biosynthesis was not observed in the wt or ΔriboB under +Fe or hFe (data not shown). During iron starvation, biomass production of the mutant displayed wt-like levels even under the low riboflavin concentration (Figure 4(a)), most likely due to the approximately fourfold decrease in biomass compared to iron sufficiency or excess that results in decreased vitamin requirement for growth. Interestingly, at low (0.1 µM) riboflavin concentration under iron starvation, siderophore production in ΔriboB decreased significantly: 82% less TAFC (P < 0.0001 vs. wt) and 96% less FC (P < 0.0001 vs. wt) compared to the wt, despite the fact that under these conditions both strains achieve the same biomass (Figure 4(b,c)). These findings indicate a crucial role for riboflavin in siderophore biosynthesis, most likely as a cofactor for ornithine-N⁵-monoxygenase SidA (Table 1), which catalyzes the initial step in siderophore biosynthesis [28].

Figure 2. Microscopic analysis of germination and growth of the deletion strains.
(a) Wild-type AF577 (wt) and deletion strains ΔriboB, ΔpanA, ΔpyrA and ΔthiB were grown in minimal liquid medium at 37°C for 24 h in the presence of increasing vitamin concentrations and analyzed by light microscopy. Higher magnification (insets, left panel) demonstrated significant swelling of ΔpyrA (P < 0.0001 vs. ΔriboB and ΔpanA) and especially ΔthiB conidia (P < 0.0001 vs. ΔriboB and ΔpanA) in the absence of vitamin supplementation compared to freshly harvested ungerminated conidia (0 h). (b) Quantification of conidial diameter of the deletion strains grown in liquid minimal medium at 37°C for 24 h in the absence of the respective vitamin.

Figure 3. Riboflavin and its derivatives FAD and FMN play a crucial role in NO detoxification and sporulation.
Wild-type AF577 (wt), ΔriboB deletion and complemented riboB⁺ strains were point-inoculated on minimal medium agar and grown at 37°C for 48 h in the presence of 2.5 µM riboflavin. Addition of (a) 5 mM nitrite (NaNO₂) selectively inhibited the growth of ΔriboB. (b) Supplementation with pantothenic acid, pyridoxine and niacin (+ vitamins) restored conidiation in ΔriboB reflected by the restoration of the greenish colony pigmentation.
Iron supplementation reduces pantothenic acid requirement of ΔpanA

Siderophore biosynthesis involves the enzymatic activity of nonribosomal peptide synthetases (NRPS), which contain 4’-phosphopantetheine, derived from pantothenic acid, as an essential prosthetic group. In A. fumigatus one such enzyme is encoded by npgA/pptA. Previous studies confirmed that NpgA function is essential for biosynthesis of TAFC and FC [29] and consequently for iron homeostasis. To characterize the effects of PanA deficiency on iron homeostasis, we analyzed the biomass production of the pantothenic acid auxotrophic mutant strain ΔpanA relative to the wt strain under iron depleted (-Fe), iron replete (+Fe, 30 µM FeSO₄) and iron excess (hFe, 5 mM FeSO₄) conditions, with either a low (0.5 µM) or a high (2.5 µM) pantothenic acid (Rib) concentration and their dry weight assessed. Under iron sufficiency and iron excess and low pantothenic acid supplementation of 0.1 µM, Δribob8 displayed a significantly decreased biomass production (+Fe: P < 0.0001 vs. wt; hFe: P < 0.0001 vs. wt) compared to the wt and to the mutant’s biomass production with high riboflavin supplementation. During iron starvation and low riboflavin supplementation, Δribob8 displayed markedly reduced biomass production of 80% during -Fe (P < 0.001 vs. wt) and 90% during +Fe (P < 0.0001 vs. wt) when supplemented with the low pantothenic acid concentration (while biomass production under high iron conditions was only mildly reduced by 28% compared to the wt). However, wt-like growth of the mutant strain was achieved by the addition of 2.5 µM pantothenic acid into the medium (Figure 5(a)). The beneficial effect of iron excess on biomass production of the pantothenic acid auxotroph was also demonstrated on solid medium (Figure 5(b)). In contrast, iron excess did not enhance growth of the riboB, pyroA and thiB null strains under limiting vitamin supplementation, further indicating the specificity of this effect in the ΔpanA mutant (data not shown). These results suggest a role for pantothenic acid in iron metabolism. In agreement, pantothenic acid is essential for activation of nonribosomal peptide synthetases, including siderophore biosynthetic SidC and SidD [30,31].

Figure 4. Decreased riboflavin availability reduces production of siderophores in A. fumigatus.

(a) Wt and ΔriboB strains were grown for 24 h at 37°C in liquid minimal medium under iron starvation (-Fe), iron sufficiency (+Fe, 30 µM FeSO₄) and iron excess (hFe, 5 mM FeSO₄) supplemented with either a low (0.1 µM) or a high (2.5 µM) riboflavin (Rib) concentration and their dry weight assessed. Under iron sufficiency and iron excess and low riboflavin supplementation of 0.1 µM, ΔriboB displayed a significantly decreased siderophore production (+Fe: P < 0.0001 vs. wt; hFe: P < 0.0001 vs. wt) compared to the wt and to the mutant’s siderophore production with high riboflavin supplementation. During iron starvation and low riboflavin supplementation, ΔriboB displayed markedly reduced (b) TAFC extracellular siderophore (P < 0.001 vs. wt) and (c) FC intracellular siderophore production (P < 0.001 vs. wt).
Hypoxia decreases pantothenic acid requirement of ΔpanA

To survive in the hypoxic necrotic tissue during infection, A. fumigatus is able to adapt to extremely low oxygen and low iron surroundings. On minimal medium agar plates, the growth defect of ΔpanA supplemented with limiting 1 µM pantothenic acid was less pronounced under hypoxic (1% oxygen) conditions compared to normoxic (21% oxygen) conditions during iron starvation (-Fe), iron sufficiency (+Fe) and iron excess (hFe, 5 mM FeSO$_4$) (Figure 6). The most remarkable phenotype was observed during iron starvation and iron sufficiency, where the ΔpanA strain under hypoxic conditions reached radial wt-like growth but completely lacked sporulation. The role of pantothenic acid during hypoxic conditions could be explained by reduced pantothenic acid requirement or by increased pantothenic uptake under these conditions [32]. In contrast, the riboB, pyroA and thiB null strains under limiting vitamin supplementation and normoxic or hypoxic iron-sufficient conditions grew like the wt (data not shown).

RibO, panA, pyroA and thiB deletion mutants are not killed by vitamin starvation

To evaluate the suitability of RiboB, PanA, PyroA and ThiB as possible drug targets, we carried out vitamin-
shift experiments to measure whether growth in the absence of vitamin causes growth arrest (i.e., inhibiting the pathway is fungistatic) or cell death (i.e., inhibiting the pathway is fungicidal). Auxotrophic strains were germinated in liquid minimal medium for 12 h in the presence of the respective vitamin (corresponding to early wt growth in the lungs during infection), and then washed repeatedly and grown for 36 h in the absence of the vitamin (corresponding to inhibition of the pathway). Finally, to determine viability, the vitamin was added to the medium for 12 h and the dry weight of the lyophilized mycelium was measured relative to non-supplemented strains. For comparison, the wt strain was starved for nitrogen (instead of vitamins as in the case of the auxotrophic mutants) and re-supplemented with nitrogen for 12 h, assuming that wt strains are capable of coping with nitrogen starvation conditions.

We found that the vitamin–starved mycelia of the \( \Delta \text{ribo}B, \Delta \text{pan}A, \Delta \text{pyro}A \) and \( \Delta \text{thi}B \) strains grew rapidly when re-supplemented with their respective vitamin, comparable to the nitrogen-starved wt (Figure 7, black bars), while without supplementation, mycelial growth remained minimal (Figure 7, grey bars). This indicates that the mycelium remained alive during the 36 h of vitamin-starvation and suggests that inhibition of these pathways will have a fungistatic (and not a fungicidal) effect.

**Riboflavin and pantothentic acid biosynthesis are required for *A. fumigatus* virulence in murine infection models**

The \( \Delta \text{ribo}B, \Delta \text{pan}A, \Delta \text{pyro}A, \Delta \text{thi}B \) strains were assessed for virulence in three immunocompromised murine models of infection: (i) a non-neutropenic pulmonary infection model in which mice were immunosuppressed with cortisone acetate (CA), (ii) a neutropenic pulmonary infection model in which mice were immunosuppressed with cyclophosphamide (CY), (iii) a neutropenic disseminated infection model in which mice were immunosuppressed with cyclophosphamide (CY) and infected via the lateral tail vein. The \( \Delta \text{ribo}B \) mutant was avirulent (100% survival) and \( \Delta \text{pan}A \) mutant was strongly attenuated (90% survival) in the non-neutropenic pulmonary model of infection, both strains were avirulent (100% survival) in the disseminated neutropenic pulmonary model of infection, and strongly attenuated in virulence (90% survival \( \Delta \text{pan}A \), 60% survival \( \Delta \text{ribo}B \)) in the neutropenic pulmonary model of infection (P < 0.0001 vs. wt), and strongly attenuated in virulence (90% survival \( \Delta \text{pan}A \), 60% survival \( \Delta \text{ribo}B \)) in the disseminated neutropenic model of infection (P < 0.0005 vs. wt) (Figure 8(a–c)). The \( \Delta \text{pyro}A \) and \( \Delta \text{thi}B \) mutants showed attenuated virulence in both pulmonary models of infection (60 to 70% survival \( \Delta \text{pyro}A \), 40% survival \( \Delta \text{thi}B \), P < 0.005 vs. wt) and in disseminated infection (P < 0.05 vs. wt) although in this last model 100% mortality of \( \Delta \text{pyro}A \) and 90% mortality of \( \Delta \text{thi}B \) was observed, possibly suggesting that there are lower levels of available pyridoxine and thiamine in the lungs compared to blood (Figure 8(a–c)). It should be noted that in the neutropenic models, profound neutropenia resolves after ~7 days and that, had an additional round of immunosuppression been administered at day +6, the virulence data may have been slightly less compelling for some of the strains. Fungal load and histopathology of infected lungs (CA, non-neutropenic host model) further reflected these results. No CFUs or visible fungal growth were found in the lungs of mice infected for 48 h with the \( \Delta \text{ribo}B \) strain. Low lung CFU counts were found in \( \Delta \text{pan}A \) (~2% of wt), increasing in \( \Delta \text{pyro}A \) (~20% of wt), and reaching wt levels in \( \Delta \text{thi}B \) (Figure 9(a)). Histopathology followed by GMS (stains fungal.
Figure 7. riboB, panA, pyroA and thiB deletion mutants are not killed by starvation of the lacking vitamin.
Deletion strains ΔriboB, ΔpanA, ΔpyroA and ΔthiB were grown in vitamin-supplemented liquid minimal medium for 12 h, washed and vitamin-starved for 36 h and finally either vitamin-re-supplemented (black bars) or not supplemented (grey bars) for another 12 h. Wild-type AF577 (wt) received the same treatment but was nitrogen-starved (instead of vitamin-starved as in the case of auxotrophic mutants) by growing without the sole nitrogen source glutamine in the 36 h starvation phase and either glutamine re-supplemented (black bars) or not supplemented for another 12 h. The biomass of the mutants was normalized to that of the wt. Biomass measurements at the end of the experiment indicate that following vitamin supplementation, the four auxotrophs remained viable and recovered their growth (black bars).

Figure 8. Riboflavin and pantothenic acid biosynthesis are essential for virulence of A. fumigatus in murine infection models.
Mouse survival curves following intranasal infection of (a) cortisone-acetate immunocompromised mice (n = 10 animals/group) and (b) cyclophosphamide-immunocompromised neutropenic mice (wt, n = 49; ΔriboB, n = 10; ΔpanA, n = 10; ΔpyroA, n = 13; ΔthiB, n = 15 animals/group). (c) Intravenous disseminated infection of cyclophosphamide-immunocompromised neutropenic mice, wt, n = 8; ΔriboB, n = 5; ΔpanA, n = 5; ΔpyroA, n = 8; ΔthiB, n = 8 animals/group).
elements black) and H&E (stains lung tissue) further corroborated these findings, showing extensive hyphal growth (arrows) and granulocyte infiltration (purple granulation-lower panel) in the airways of mice infected with the wt strain, which was slightly attenuated following ΔthiB infection, greatly reduced following ΔpyroA and ΔpanA infection and totally absent in the ΔriboB–infected mice (Figure 9(b)).

Discussion
A central problem in the development of antifungals is the lack of suitable drug targets not found in humans. In this report, using the pathogenic mold A. fumigatus as our model, we analysed four fungal-specific pathways involved in the biosynthesis of riboflavin (vitamin B2), pantothenic acid (vitamin B5), pyridoxine (vitamin B6) and thiamine (vitamin B1) as potential drug targets.

We deleted genes encoding key enzymes in each of these pathways (riboB, panA, pyroA and thiB), which resulted in auxotrophy. Our underlying hypothesis was that the vitamins produced by these pathways might not be available for fungal growth in the host, resulting in dependence on endogenous biosynthesis. Past work in A. fumigatus has shown this to be the case for folate (Vitamin B9) biosynthesis. Deletion of pabA, encoding PABA synthase, catalysing an early step in folate biosynthesis, abrogated virulence in infected mice, designating this pathway as an excellent candidate for the development of novel antifungals [33]. Antibacterial sulfa drugs that inhibit dihydropteroate synthase in the folic acid biosynthetic pathway already exist and show weak antifungal activity [34]. Recent efforts have focused on developing antifungals to target other enzymes in this pathway [35].

Our main finding was that deletion of riboB and panA abolished or strongly attenuated virulence (depending on the infection model), pyroA deletion attenuated virulence and loss of thiB weakly reduced virulence. Virulence was tested in three models of murine infection that recapitulate the most common types of invasive aspergillosis infections [36]. We modelled invasive pulmonary

Figure 9. Deficiency in riboflavin, pantothenic acid and pyridoxine biosynthesis significantly reduce A. fumigatus lung burden in cortisone-acetate immunocompromised mice.
(a) Lung fungal load and (b) lung histology of intranasally-infected cortisone-acetate immunocompromised mice (n = 5 animals/group), after 48 h of infection. Staining was done with GMS (stains fungus black, see black arrows) or H&E lung stain. Bar = 200 μm.
Aspergillus in the neutrophilic host using cyclophosphamide and in the non-neutrophilic compromised host using cortisone acetate. Disseminated aspergillosis, which frequently occurs in the neutrophilic host, was performed by intravenous infection. Our results suggest that there are insufficient levels of riboflavin and pantothenic acid in the infected host to support growth of the ΔriboB and ΔpanA mutants. In contrast, the ΔpyroA and ΔthiB mutants need only very low levels (0.1–2.5 nM) of external pyridoxine and thiamine for growth, which are available in the host at far higher concentrations [37,38], most likely explaining their milder influence on virulence. Notably, the virulence behaviour of the four mutants matched their growth pattern on blood agar plates: in contrast to the ΔpyroA and ΔthiB strains, ΔriboB and ΔpanA mutant strains were unable to grow on blood agar without vitamin supplementation. Virulence studies with auxotrophic mutants also serve to describe nutrient availability in different host niches. The strongly attenuated virulence of ΔriboB and ΔpanA mutants in all tested virulence models demonstrates that both riboflavin and pantothenic acid biosynthesis are not only essential for invasion (intranasal infection model) but also during dissemination (intravenous infection model). In contrast, lysine biosynthesis was previously found to be essential exclusively for invasion but not during dissemination [10,11]. Virulence analysis of auxotrophs was previously tested in the closely related Aspergillus nidulans during the 1970s. Interestingly, they gave slightly different results. As found here for A. fumigatus, thiamine auxotrophy did not reduce virulence. However, in A. nidulans, riboflavin auxotrophy attenuated but did not abolish virulence whereas pyridoxine auxotrophy abolished virulence [39]. However, these studies were performed with mutagenized strains in which the target genes were not deleted and they lacked the complemented strains as controls.

Riboflavin is the precursor of flavin mononucleotide (FMN) and flavin adenine nucleotide (FAD) and as such is an essential cofactor for many flavoprotein-catalyzed physiological processes. Remarkably, A. fumigatus encodes approximately 238 flavoproteins (Supplementary Table 7) involved in central metabolic pathways and regulatory circuits (Table 1) including for example NO-detoxification (flavohemoglobinins FhpA and FhpB), siderophore biosynthesis (SidA), reductive iron assimilation (metalloreductase FreB), amino acid biosynthesis, ergosterol biosynthesis, vitamin biosynthesis, and secondary metabolism. In contrast, S. cerevisiae possesses only 68 flavoproteins [40]. The greatly increased number of flavoproteins in A. fumigatus compared to S. cerevisiae might indicate a higher demand for riboflavin. We confirmed here the importance of riboflavin for NO-detoxification and siderophore biosynthesis in A. fumigatus. In contrast to NO-detoxification, which appears to be dispensable for virulence of A. fumigatus [41], siderophore biosynthesis has been shown to be essential for virulence of several animal and plant pathogenic fungi [42]. Similarly, pantothenic acid is of central importance for primary metabolism as it is essential for coenzyme A biosynthesis and consequently for fatty acid metabolism, carbohydrate metabolism and tricarboxylic acid cycle [43]. Moreover, it is an essential prosthetic group for polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) including the siderophore biosynthetic NRPSs. The impact of PKSs and NRPSs is reflected by the avirulence of the A. fumigatus mutant lacking the pantothenic acid dependent enzyme phosphopantetheinyl transferase PptA/NpgA [30]. The requirement of PptA/NpgA and consequently pantothenic acid for activation of NRPSs including the siderophore biosynthetic NRPSs SipD and SipC is consistent with the beneficial effect of iron supplementation on biomass production of ΔpanA during limited pantothenic acid supplementation (Figure 4).

How suitable are the riboflavin and pantothenic acid biosynthetic pathways for development as drug targets? The riboflavin biosynthesis pathway contains many genes absent in humans, including rib3, rib4, rib5, rib7 and rib1/riboB described here (see supplementary Table 3). We show that deletion of riboB in A. fumigatus leads to avirulence in cortisone-compromised mice and in neutropenic mice with disseminated infection. Lung CFU enumeration and histology show complete clearance of the fungus from the lungs 48 h after infection. Riboflavin auxotrophy also leads to avirulence in Candida albicans [6], Histoplasma capsulatum [23] and in many species of gram-negative bacteria including Mycobacteria that lack a riboflavin uptake system and are therefore totally dependent on endogenous biosynthesis [44]. Therefore, this pathway is particularly suitable for the development of antimicrobials. Based on detailed enzymatic and structural data, substrate inhibitors have been developed for the last two steps of riboflavin biosynthesis, catalysed by lumazine synthase and riboflavin synthase, respectively. To the best of our knowledge, in vivo use of these compounds has not been described, suggesting there were pharmacological problems in their development [45,46].

The pantothenic acid biosynthesis pathway is also rich in possible antimicrobial targets including pan2, pan5, and pantothenate synthase pan6/panA (see supplementary Table 4) [47]. A major effort has been made to develop pantothenate synthase inhibitors active against Mycobacterium tuberculosis, as this enzyme is essential for virulence [48]. Although several showed in
vitro activity, none was described as acting in vivo [49]. Subsequent CoA synthesis from pantothenate is controlled by pantothenate kinase, encoded by the essential gene cab1 (S. cerevisiae)/panK (A. nidulans) [50]. Several pantothenic acid analogues that inhibit this enzyme have shown antibacterial activity in vitro and in vivo [47] but none were tested for fungal infections.

Two drawbacks should be noted regarding the suitability of the enzymes RiboB and PanA as antifungal targets—first, our in vitro vitamin-shift analysis suggests that inhibiting these enzymes will have a fungistatic rather than fungicidal action. Second, inhibition of a single cellular target often results in mutations leading to resistance. Nevertheless, these problems can be circumvented by developing inhibitors that (i) react with the target enzyme to generate toxic products or (ii) block additional essential fungal targets, and (iii) by using drug combinations [3].

Future work needs to test existing inhibitors of riboflavin and pantothenic acid biosynthesis for activity in pathogenic fungi both in vitro and in vivo. Crystallographic data of key fungal enzymes (RiboB, PanA etc.) needs to be generated and used to plan more specific and potent antifungal inhibitors. Compound library screens can make use of the fact that inhibitors specifically acting on these pathways are neutralized by the addition of exogenous riboflavin and pantothenic acid, respectively [51].

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