



Cite this: *Metallomics*, 2017, 9, 1655

The cytochrome *b*₅ CybE is regulated by iron availability and is crucial for azole resistance in *A. fumigatus*

Matthias Misslinger,^a Fabio Gsaller,^b Peter Hortschansky,^c Christoph Müller,^d Franz Bracher,^d Michael J. Bromley^b and Hubertus Haas^{b,*}

Cytochrome P450 enzymes (P450) play essential roles in redox metabolism in all domains of life including detoxification reactions and sterol biosynthesis. The activity of P450s is fuelled by two electron-transferring mechanisms, heme-independent P450 reductase (CPR) and the heme-dependent cytochrome *b*₅ (CYB5)/cytochrome *b*₅ reductase (CB5R) system. In this study, we characterised the role and regulation of the cytochrome *b*₅ CybE in the fungal pathogen *Aspergillus fumigatus*. Deletion of the CybE encoding gene (*cybE*) caused a severe growth defect in two different *A. fumigatus* isolates, emphasising the importance of the CB5R system in this pathogen, while the non-essentiality of *cybE* indicates the partial redundancy of the CPR and CB5R systems. Interestingly, the growth defect caused by the *cybE* loss of function was even more drastic in *A. fumigatus* strain Afs77 compared to strain A1160P+ indicating a strain-dependent degree of compensation, which is supported by azole resistance studies. In agreement with CybE being important for the assistance of the ergosterol biosynthetic P450 Cyp51, deletion of *cybE* decreased resistance to the Cyp51-targeting antifungal voriconazole and caused accumulation of the ergosterol pathway intermediate eburicol. Northern analysis indicated that CybE deficiency leads to the compensatory transcriptional upregulation of Cyp51-encoding *cyp51A* and CPR-encoding *cprA*. Overexpression of *cybE* did not affect azole resistance suggesting that CybE activity is not rate limiting. Expression of *cybE* was found to be repressed during iron starvation by the iron-regulatory transcription factor HapX demonstrating iron dependence of CybE not only at the level of enzyme activity but also at the level of gene expression.

Received 7th April 2017,
Accepted 31st July 2017

DOI: 10.1039/c7mt00110j

rsc.li/metallomics

Significance to metallomics

Cytochrome P450 enzymes (P450) are involved in oxidative metabolism and biosynthesis in all kingdoms of life. The required electrons for this redox chemistry are provided by an iron-dependent and an iron-independent system. With a focus on the iron-dependent cytochrome *b*₅, this study gives new insights about the fine-tuning of electron transfer systems dependent on iron availability.

Introduction

Cytochrome P450 enzymes (P450) are found in all domains of life. They play important roles in xenobiotic metabolism/biotransformation,¹ secondary metabolism² and primary metabolism such

as biosynthesis of fatty acids, vitamins^{3,4} and sterols.⁵ Although P450 amino acid sequences are extremely diverse between species, their structures are highly conserved. This structural conservation allows all P450s to bind the cofactor heme, which is essential for their function.⁶ In addition to the structural conservation, members of this family share the catalytic property to activate molecular oxygen to incorporate one molecule of oxygen into a substrate, while the other is reduced to water by NAD(P)H.⁴ This reductive activation is highly dependent on free electrons and on the co-factor heme of the respective cytochrome P450.⁶ The heme-bound iron is a crucial factor for this chemistry, as it can adopt different oxidation states and hence serves as an electron acceptor and donor.⁷ In many species including the yeast *Saccharomyces cerevisiae*, the two

^a Division of Molecular Biology, Biocenter, Medical University of Innsbruck, Innrain 80, 6020 Innsbruck, Austria. E-mail: hubertus.haas@i-med.ac.at

^b Manchester Fungal Infection Group, Institute of Inflammation and Repair, University of Manchester, Manchester, UK

^c Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology (HKL), Jena, Germany

^d Department of Pharmacy, Center for Drug Research, Ludwig-Maximilians-University Munich, Munich, Germany



electrons, which are required for a P450 reaction, are provided by two partially redundant systems.^{8,9} In one system, electrons are passed from NADPH by a cytochrome P450 reductase (CPR) to the respective P450. In the second system, electrons are supplied to P450s by the cytochrome *b*₅ (CYB5)/NADH cytochrome *b*₅ reductase (CB5R) system. It is speculated that CYB5 optimises the kinetics of P450 reactions by allosteric enhancement and by acting as an electron store. In the latter mechanism, the second electron needed for P450 reactions is supplied by CYB5 immediately after the activation of oxygen.¹⁰ This facilitation of electron supply increases the formation of reaction products and decreases the release superoxide.

CYB5 is also involved in P450-independent reactions as an electron shuttle in systems such as the electron transfer to microsomal desaturases, which are important for unsaturated fatty acid synthesis.^{11,12} CYB5 also contains heme as a prosthetic group, which is involved in shuttling electrons to the respective target.^{13,14}

Ergosterol is the predominant sterol in fungal membranes.⁵ One step in ergosterol biosynthesis includes sterol-C14 α demethylation, which is performed by the P450 sterol-C14 α demethylase CYP51.¹³ This enzyme is the target of the azole class of antifungals, which bind to CYP51, and inhibit its function and thereby the biosynthesis of ergosterol, resulting in lethality.⁵ In two yeast species, *S. cerevisiae* and *Candida albicans*, individual inactivation of either CPR or CYB5 was not lethal and decreased but did not block ergosterol biosynthesis.^{14–16} In contrast, simultaneous inactivation of both CYB5 and CPR was lethal,^{14,17} which corroborates the partial redundancy of these systems in these yeast species. In addition, the CYB5/CB5R system was found to fully support yeast CYP51 *in vitro*.¹³ Due to decreased CYP51 activity, loss of CPR leads to increased azole susceptibility, and overexpression of the CYB5 encoding gene in such a setting increased azole resistance in contrast to the CPR knockout, which emphasises partial redundancy of the CYB5/CB5R and CPR systems in electron transport to CYP51 in yeast.¹⁴

The filamentous fungus *Aspergillus fumigatus* is an opportunistic human pathogen and is the major cause for life-threatening invasive aspergillosis.¹⁸ Immunocompromised patients are especially at high risk for being infected. One of the most common treatments of fungal infections including aspergillosis is the inhibition of the sterol-C14 α -demethylase CYP51 (termed Erg11 in yeast species) with triazole-type antifungals.^{19–21} The *A. fumigatus* genome encodes two CYP51 enzymes termed Cyp51A and Cyp51B.²² Both are able to perform sterol-C14 α demethylation with similar activity.^{23,24} However, most mechanisms described for azole resistance are linked to Cyp51A.^{21,25}

The *A. fumigatus* genome, similar to other filamentous fungal genomes, comprises 77 genes encoding for P450s.^{2,26} This quantity is striking compared to the widely used model organism *S. cerevisiae*, which only possesses three P450s: the sterol-C14 α -demethylase CYP51/Erg11 and the sterol-C22-desaturase CYP61/Erg5, which are both involved in ergosterol biosynthesis, and the ditryptophan hydroxylase CYP56, which is important for yeast sporulation.¹³ In contrast to *S. cerevisiae*, mould species like *A. fumigatus* produce a wide range of secondary metabolites,

the biosynthesis of which requires numerous P450-dependent oxygenation reactions.²⁷ Consequently, the expansion of P450s in moulds compared to *S. cerevisiae* is most likely linked to their complex secondary metabolism. The *A. fumigatus* genome encodes one putative CYB5, termed CybE (AFUA_2G04710), and two putative CPRs, termed CprA (AFUA_6G10990) and CprB (AFUA_2G07940), which are potentially involved in the biosynthesis of ergosterol and secondary metabolites.

Iron is essential for almost all organisms, including the heme function in P450s and CYB5. Iron acquisition and the expression of iron-consuming genes has to be tightly regulated to keep the balance between iron toxicity and iron shortage.²⁸ In *A. fumigatus*, this includes gene regulation by the iron-responsive transcription factor HapX, which represses iron-consuming pathways during iron starvation in order to spare iron and activates the respective genes during iron excess.²⁹ Adaptation to iron starvation including high-affinity acquisition *via* siderophores and HapX-mediated transcriptional reprogramming has been demonstrated to be crucial for the virulence of *A. fumigatus*.³⁰ This iron-responsive reprogramming also involves the SREBP (sterol regulatory element-binding protein)-type regulator SrbA, which links the control of iron metabolism, ergosterol biosynthesis and adaptation to low oxygen conditions.³¹

In this study, we characterised the function of CybE in *A. fumigatus* *via* gene inactivation and overexpression, including its role in ergosterol biosynthesis, demonstrating its regulation by iron availability *via* the transcription factor HapX.

Experimental

Growth conditions

In liquid cultures, strains were grown at 37 °C and 200 rpm in *Aspergillus* minimal medium (MM),³² containing 1% glucose (carbon source), 20 mM glutamine (nitrogen source) and 30 μ M FeSO₄ (if not otherwise indicated). For plate assays, MM agar or agarose was used (agarose for –Fe conditions to avoid iron contaminations). Plates were grown for 48 h at 37 °C. For low oxygen experiments, strains were grown for 48 h at 37 °C with 1% O₂ and 5% CO₂.

A. fumigatus strains, generation of *cybE* deletion mutants, *cybE* inducible strains and reconstitution of Δ *cybE*

The coding sequence of *cybE* (AFUA_2G04710) was replaced by a hygromycin (*hph*) resistance conferring cassette in the AfS77 and A1160P+ strains. The deletion construct was generated by fusion PCR following an approach previously described.³³ In the first step, around 1 kb of the *cybE* 5'-flanking region and 1 kb of the 3'-flanking region were amplified by PCR using primers the AACCCATCCTGTCTGACCGA & TAGTCTGTACC GAGCCGGCGGTTTATCTGGATTGGTGACG and GCTCTGAACG ATATGCTCCCGCAAGGGGAAAGGAGGTCTG & GGCGTCATCCC TTCCATTGA. Subsequently, fragments were linked to a hygromycin resistance cassette (amplified with the primers CCGGCTCGGTAA CAGAACTAACGGCGTAACCAAAAGTCAC and GGGAGCATATCGTT CAGAGCTCTTGACGACCGTTGATCTG) by PCR fusion using the



A. fumigatus isolates were used for this study, AfS77³⁶ and A1160P+.³³ The strains used in this study are listed in Table 1.

To reconstitute $\Delta cybE$, plasmid pcybE^{REC}, comprising a pyrithiamine resistance cassette, was generated. The backbone of the pyrithiamine resistance cassette carrying plasmid pSK275 (syn. pME3024³⁴) was amplified using the primers ACCGGTCAAA GCTAAAGAGG and ACTAGTTCTAGAGCGGCCG.

The *cybE* coding sequences including 1.5 kb 5'- and 1.9 kb 3'-flanking regions were amplified with the primers CGGCCGC TCTAGAACTAGTCTGCTCTGTGGCCTGTGATA and CCTCTTTA GCTTTGACCGGTCGCGTGCAGATAAAGATCAA. Both fragments were connected *via* Gibson Assembly[®].

To generate a strain in which *cybE* is inducible by the addition of xylose to the growth medium (*cybE^{xyIP}*), the promoter of *cybE* was separated from the coding sequence by a hygromycin cassette and a xylose inducible *xyIP* promoter.³⁵ The construct was generated using the NEBuilder[®] kit (New England Biolabs), with the primers MM118 (AATTCGAGCTCGGTACAGCGGAAGAC CATTC), MM119 (TACCTAGGTGTGTAGAAATTATTCGGTTT), MM120 (TCTACACACCTAGGTACAGAAGTCC), MM121 (GCGGA CATGGTTGGTTCTTCGAGT), MM122 (AACCAACCATGTCCGCC TCCAAG), MM123 (GCCAAGCTTGCATGCCCACTCCACTGTTGA GAG), and linear pUC19 (amplified with the primers GGCATG CAAGCTTGGCGT and GTACCGAGCTCGAATTCAGT) as a plasmid backbone. MM118/119, MM120/121 and MM122/123 were used to amplify the 5'-homologous region, a hygromycin-*xyIP* cassette and the 3'-homologous region, respectively.

Linear knockout- and promoter exchange constructs, as well as pcyb^{REC}, were transformed into *A. fumigatus* protoplasts and integrated into the genome by homologous recombination. Integration was proven by Southern blot analysis (Fig. 1). pcyb^{REC} restored the wildtype phenotype in the $\Delta cybE$ strain, just as *cybE* induction did in the *cybE*^{xyIP} strain (Fig. 1). Two different

RNA isolation and northern blot analysis

RNA was isolated using TRI Reagent[®] (Sigma) according to the manufacturer's manual. 10 µg of RNA was used for electrophoresis on 2.2 M formaldehyde agarose gels and subsequently blotted onto Hybond N membranes (Amersham). DIG labelled probes were amplified by PCR.

Southern blot analysis

DNA was isolated by PCI extraction and isopropanol precipitation. To confirm the gene-specific restriction pattern of the genetic manipulations, the DNA was digested with PstI (AfS77 strains) or BamHI (A1160P+ strains). The resulting restriction fragments were separated on an agarose gel and transferred to a Hybond N membrane (Amersham) by capillary blotting with NaOH. The deletion of *cybE* caused the deletion of a PstI site in the CDS and the insertion of a BamHI site. The *xyIP*-promoter insertion elongated the space between the PstI sites. Integration of *pcyB*^{REC} generated a second 5'-region of *cybE*. These differences in fragment size were detected with a DIG-labelled probe binding to the 5'-region of *cybE*. A successful genetic manipulation leads to the following fragments: wt PstI 1752 bp, $\Delta cybE$ PstI 3368 bp, *cybE*^{xyIP} PstI 2641 bp, wt BamHI 8107 bp, $\Delta cybE$ BamHI 5804 bp, *cybE*^{REC} BamHI 5804 and 10 521 bp.

Susceptibility tests

For diffusion tests, conidia were mixed with 25 mL of MM to a final concentration of 10^6 conidia per mL. These plates with 25 mL of MM were poured, and a paper disc was placed in the middle or a hole was pricked out, respectively. For oxidative stress tests, the hole was filled with 100 μ L H_2O_2 (34 mg mL⁻¹).

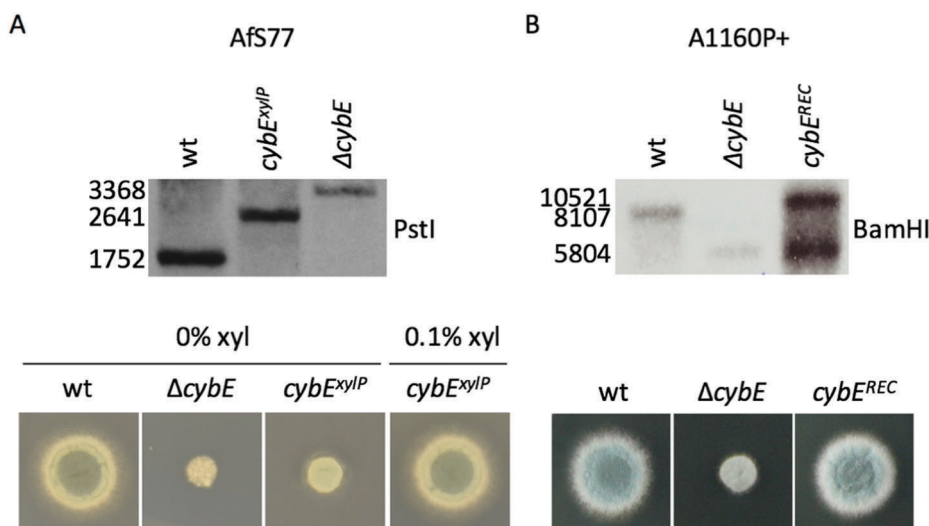


Fig. 1 Southern blot analysis and growth of *cybE* mutant strains on solid iron-replete minimal medium (MM). Strains were generated in the two *A. fumigatus* isolates: (A) AfS77 and (B) A1160P+. $\Delta cybE$ strains are *cybE* gene deletion strains; in strain *cybE*^{REC}, the *cybE* gene was re-integrated at the *cybE* deletion locus in $\Delta cybE$; in strain *cybE*^{xyI}, *cybE* expression is controlled by the xylose-inducible *xyI* promoter. Strains have been validated using Southern blot analysis revealing the expected pattern (see the Experimental section). $\Delta cybE$ and *cybE*^{xyI} under non-inducing conditions display reduced growth, while *cybE*^{REC} and *cybE*^{xyI} under inducing conditions (0.1% xylose) show growth similar to wt.

Table 1 Strains used for this study

Strain	Genotype	Ref.
wt, A1160P+	A1160, $\Delta akuB::pyrG^+$	33
$\Delta cybE$, A1160P+	A1160P+, $\Delta cybE::hph$	This study
$cybE^{REC}$, A1160P+	A1160P+, $\Delta cybE::hph$, $pcybE^{REC}$, $ptrA$	This study
wt, AfS77	ATCC46645, $\Delta akuA::loxP$	36
$\Delta cybE$, AfS77	AfS77, $\Delta cybE::hph$	This study
$\Delta cybE^{xyIP}$, AfS77	AfS77, $pcybE::hph$, p_{xyIP}	This study
$\Delta srbA$	AfS77, $\Delta srbA::hph$	This study

For antifungal susceptibility tests, the paper disc was drenched with voriconazole (10 μ L of a 320 μ g mL⁻¹ solution in DMSO) or with terbinafine (10 μ L of a 100 μ g mL⁻¹ solution in DMSO).

Sterol measurements with gas chromatography ion trap mass spectrometry (GC-IT-MS)

Sterols were analysed as their corresponding trimethylsilyl (TMS) ethers. The sterols were identified by their relative retention time and mass spectra.^{37–39} Strains were grown in MM for 24 h at 37 °C and 200 rpm. Mycelia were harvested, freeze-dried, ground and dissolved in 2 M NaOH. Subsequent procedures were performed according to Müller *et al.*³⁸ The

content for each sterol was calculated according to Müller and Bracher.⁴⁰ The samples were measured in biological triplicates.

Results and discussion

Deletion of *cybE* causes severe growth defects in *A. fumigatus*

To analyse the function of cytochrome *b*₅, mutant strains were generated lacking *CybE* (AFUA_2G04710), here referred to as $\Delta cybE$, by replacing the encoding gene *cybE* with a hygromycin resistance cassette, as described in the Experimental section. This gene deletion was performed in the *A. fumigatus* isolate A1160P+,³³ which is an *akuB::pyrG* derivative of CEA10 (termed wt A1160P+). To confirm the *cybE* specificity of this genetic manipulation, the *cybE* gene was reinserted in the $\Delta cybE$ mutant strain in single copy at the *cybE* locus yielding *cybE*^{REC} (see the Experimental section). Correct integration of the deletion construct and the reconstitution construct, respectively, was proven by PCR analysis (not shown) and Southern blot analysis (Fig. 1). In addition, a $\Delta cybE$ strain was generated in AfS77,³⁶ which is an *akuA::loxP* derivative of ATCC46645 (termed wt AfS77). Gene deletion in both genetic backgrounds caused a clear reduced growth phenotype. Reinsertion of *cybE* in $\Delta cybE$ in the A1160P+



Fig. 2 Deletion of *cybE* caused severe growth defects in solid and liquid media. (A) 10⁴ spores were spotted in the solid complex medium (CM) containing 0% (*cybE*^{xyIP} repressing conditions) or 0.1% xylose (*cybE*^{xyIP} inducing conditions). (B) 10⁸ spores were inoculated in 100 mL minimal medium (MM) containing no (–Fe), 30 μ M (+Fe) and 5 mM iron. *cybE*^{xyIP} was grown under *cybE* repressing (0% xylose) and overexpressing (1% xylose) conditions. Error bars indicate the standard deviation. Asterisks indicate the level of significance according to the *p*-values of an unpaired two-tailed Student's *t*-test: * for *p* = 0.054, * for *p* < 0.05, ** for *p* < 0.01 and *** for *p* < 0.001. Cultures were analysed in triplicate. (C and D) 10⁴ spores were spotted on solid MM containing –Fe, +Fe and 10 mM iron. Plates were incubated for 48 h at 37 °C under normal oxygen conditions (C) and in parallel under low oxygen conditions (D, 1% O₂, 5% CO₂). In plate cultures, the mycelial density of the colonies is significantly lower during –Fe compared to +Fe, and therefore the radial growth is not informative for the growth rate in this case. Iron starvation is reflected by the decreased sporulation.



genetic background (strain *cybE^{REC}*) restored the wt phenotype, which proves that the growth defect is *cybE* deletion specific and not an off-target effect (Fig. 1). To study the effects of *cybE* overexpression, the gene was expressed in AfS77 under the control of the xylose-inducible *xyIP*-promoter.³⁵ Growth of this strain, termed strain *cybE^{xyIP}*, under non-inducing conditions (0% xylose) resulted in decreased growth, albeit less extreme compared to *cybE* deletion, indicating decreased *cybE* expression compared to the wt strain (Fig. 1). Induction of *cybE* gene expression (0.1% xylose) again fully restored the wt phenotype.

In both genetic backgrounds (A1160P+ and AfS77), the deletion of *cybE* led to severe growth defects on plates, as well as in liquid cultures in both minimal (MM) and complex (CM) growth media (Fig. 2A–C). Interestingly, the growth defect caused by *cybE* deletion was more severe in the AfS77 genetic background compared to A1160P+.

Cybe has a heme-binding domain and is supposed to be a hemoprotein like other cytochrome *b₅* proteins.⁹ Consequently, it requires iron for proper function. Therefore, we compared

the growth of wt, $\Delta cybE$ and *cybE^{xyIP}* strains under conditions of different iron availabilities. Despite the growth defect, both the $\Delta cybE$ and uninduced *cybE^{xyIP}* strains responded to starvation and excess of iron similar to the wt strain (Fig. 2B–D). This underpins an important role of Cybe independent of iron availability.

Taken together, the severe growth defect caused by Cybe deficiency in two different *A. fumigatus* isolates emphasises the importance of the CB5R system in this species while the non-essentiality of *cybE* indicates the partial redundancy of the CPR and CB5R systems. Moreover, the difference in the degree of growth impairment in the two *A. fumigatus* strains demonstrates the strain-dependent degree of compensation.

Cybe deficiency increases susceptibility to voriconazole, terbinafine, oxidative stress, and cellular accumulation of the ergosterol biosynthetic pathway intermediate eburicol

The ergosterol biosynthetic P450 enzyme sterol-C14 α -demethylase, termed Cyp51, is the target for triazole-type antifungals such as

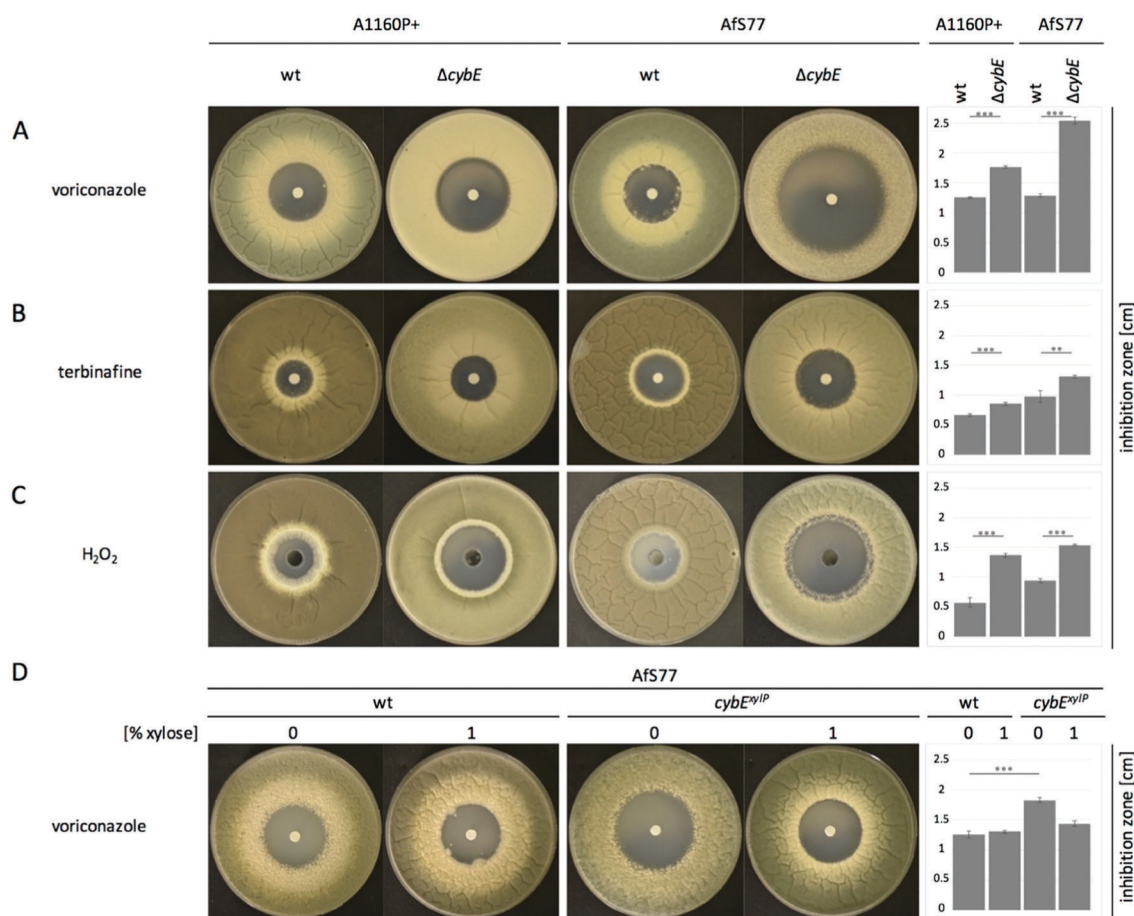


Fig. 3 Cybe deficiency increases the susceptibility to voriconazole, terbinafine and H₂O₂. For inhibitor diffusion assays, plates containing 25 mL of MM were inoculated homogeneously with 2×10^7 spores. (A) 10 μ L of voriconazole (320 μ g mL⁻¹) or (B) terbinafine (100 μ g mL⁻¹) was administered onto a filter paper disk in the centre. (C) For oxidative stress tests, 100 μ L of H₂O₂ (34 mg mL⁻¹) was added to a well in the centre. (D) Voriconazole resistance was also examined under *cybE* repressing and overexpressing conditions. Pictures were taken after 48 h of incubation at 37 °C. From three biological replicates, the mean size of the inhibition zone, which reflects the compound susceptibility, is shown as bar graphs on the right of each row. Error bars indicate the standard deviation. Significance levels are indicated as asterisks according to the *p*-values of an unpaired two-tailed Student's *t*-test: * for *p* < 0.05, ** for *p* < 0.01 and *** for *p* < 0.001.

in Fig. 5C, did not increase voriconazole resistance of the *cybE^{xyIP}* strain compared to the wt strain (Fig. 3D), which indicates that CybE activity is not a limiting factor for the Cyp51 activity in *A. fumigatus*, even under triazole stress. These data also indicate that mutations leading to gain of function of CybE are not expected to play a role in the development of resistance to triazoles in clinical settings.

The increase in the voriconazole susceptibility caused by loss of CybE indicates the role of this enzyme in sterol biosynthesis, perhaps *via* Cyp51. This is corroborated by the analysis of the cellular sterol content of wt and $\Delta cybE$ strains (Fig. 4). In line with CybE assisting Cyp51 enzyme activity, CybE deficiency caused a 0.76-fold decrease in the cellular ergosterol content and an 18-fold increased accumulation of eburicol, the substrate of Cyp51. Such a change of the cellular sterol profile is also caused by deletion of one of the two Cyp51-encoding genes or by triazole treatment.⁵ Reintegration of *cybE* into $\Delta cybE$ (strain *cybE*^{REC}) restored the wt sterol profile (Fig. 4), proving that the changes observed are indeed caused by gene deletion.

The viability and ergosterol production of CybE-deficient strains indicate that alternative systems are able to compensate for this defect. In agreement, CprA has been shown to be able to

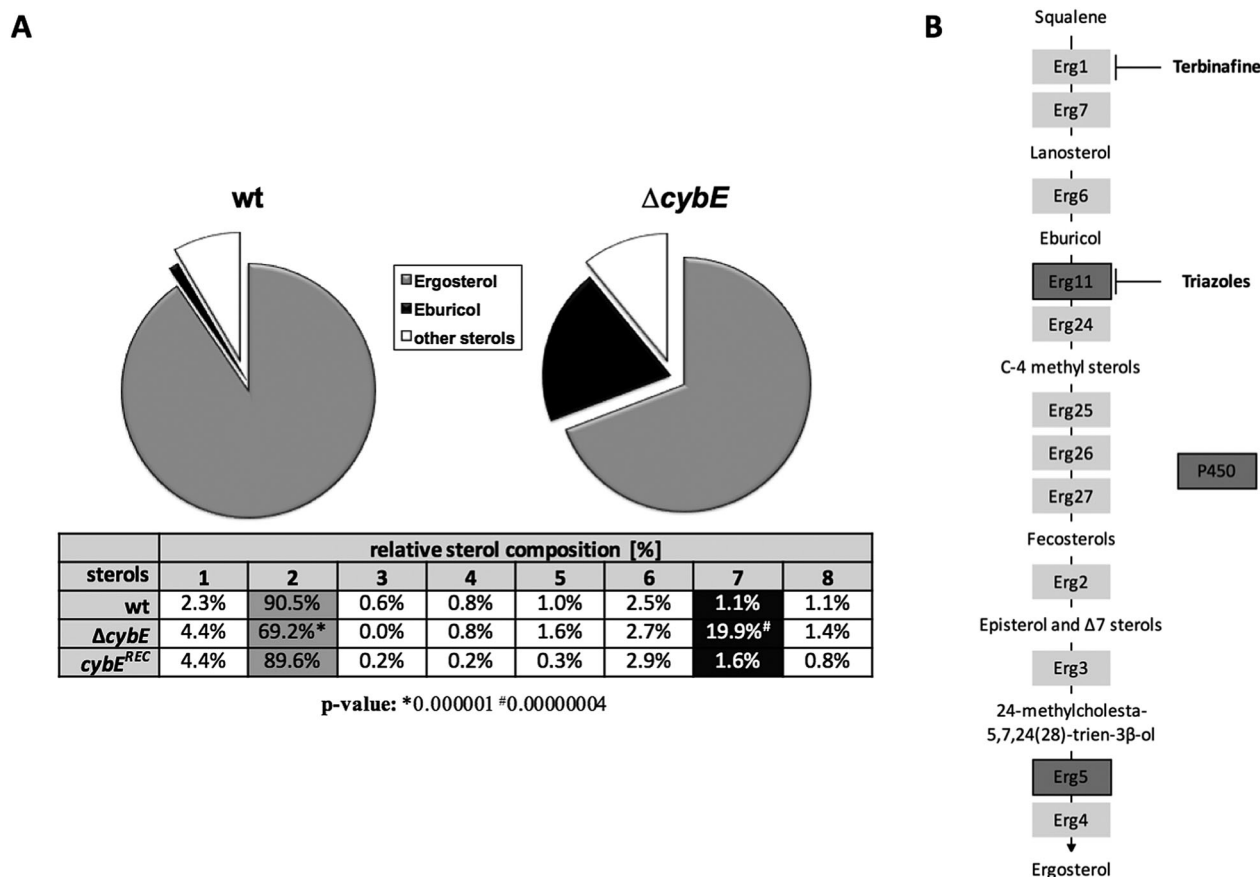


Fig. 4 CybE deficiency decreases the cellular ergosterol content and increases accumulation of eburicol. (A) Sterol levels of strains incubated in MM for 24 h at 37 °C and at 200 rpm were quantified by GC-MS. The sterol content has been normalised to total sterol levels. Samples were assessed in biological triplicates. Sterols: 1, ergosta-5,7,9(11),22-tetraen-3 β -ol; 2, ergosterol; 3, 5,6-dihydroergosterol; 4, ergosta-5,7,24(28)-trien-3 β -ol; 5, episterol; 6, lanosterol; 7, eburicol; and 8, 4,4-dimethylergosta-8,24(28)-dien-3 β -ol. Significance levels were calculated by employing an unpaired two-tailed Student's *t*-test. (B) The ergosterol biosynthesis pathway. The two P450 enzymes and the targets of terbinafine and triazoles are indicated.

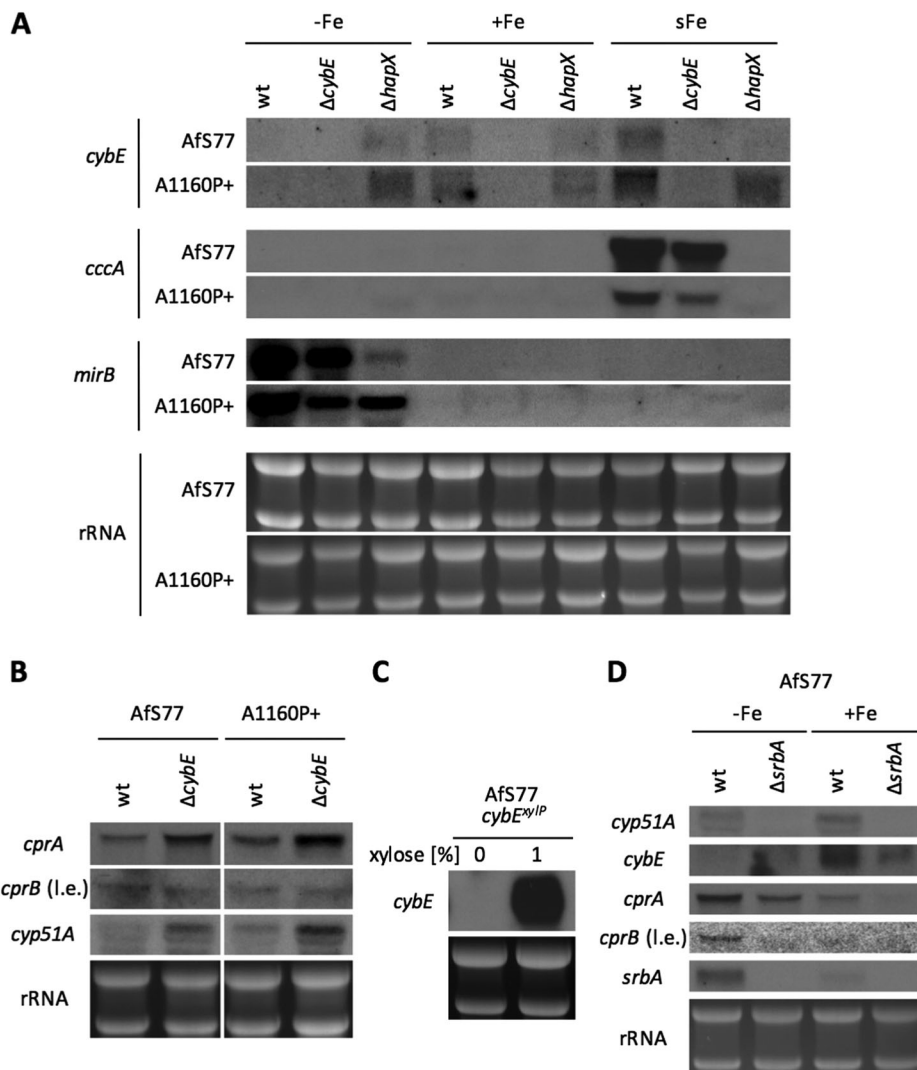


Fig. 5 *cybE* expression is iron-regulated and its deletion triggers compensatory transcriptional changes. RNA was isolated from the strains grown for 20 h in MM containing no iron (–Fe) or 30 μ M iron (+Fe) at 37 °C. $\Delta cybE$ strains were grown for 40 h to ensure similar biomass and growth phase. For the iron shift (sFe), iron was added to a final concentration of 30 μ M for 30 min after pregrowth for 20 h (wt)/40 h ($\Delta cybE$) under iron starvation conditions. Ethidium bromide-stained ribosomal RNA (rRNA) of the gels is shown as a control for the loading and quality of RNA. (A) HapX-mediated iron regulation of *cybE* transcript levels. *cccA* and *mirB* were included as controls for correct iron conditions and genetic backgrounds (see text). (B) Deletion of *cybE* causes compensatory upregulation of genes, which are involved in P450 enzyme reactions. In contrast to the other genes, *cprB* transcripts were detectable only after long exposure (“l.e.”) indicating the low level of expression. (C) Expression of *cybE* can be repressed and induced by xylose addition to the medium in *cybE*^{xyIP}. (D) *SrbA* is involved in the regulation of P450 associated gene expression.

The transcription factor *SrbA* regulates not only *cyp51* expression but also Cyp51 associated genes

SrbA is a SREBP-type transcriptional activator that plays an important role in the regulation of sterol biosynthesis, and adaptation to hypoxia and iron starvation in *A. fumigatus*.^{51,52} One of the *SrbA* target genes is *cyp51A* and, consequently, *SrbA* deficiency leads to a dramatically increased susceptibility to triazole-type antifungals.^{53,54} In agreement, northern blot analysis confirmed the transcriptional downregulation of *cyp51A* in response to *SrbA* deficiency in the AfS77 background during both iron sufficiency and starvation (Fig. 5D). Furthermore, *SrbA* deficiency was found to result in the downregulation of transcript levels of *cprA* during iron starvation and sufficiency,

cybE during iron sufficiency, and *cprB* during iron starvation (Fig. 5D). These data indicate that these Cyp51-assisting protein-encoding genes belong to the *SrbA* regulon in order to support the coordination of sterol biosynthesis. In accordance with previous findings,³¹ *srba* was transcriptionally upregulated during iron starvation (Fig. 5D). Interestingly, also *cprA* and *cprB* were found to be transcriptionally upregulated during iron starvation, while *cybE* was downregulated under this condition as already discussed above (Fig. 5D). The inverse regulation of *cybE* and *cprA/B* genes in response to iron availability indicates the cellular optimisation of partially alternative iron-dependent and iron-independent systems to iron availability.



CyB is not essential under low oxygen conditions

A. fumigatus can cope with extremely low oxygen conditions, which is important to adapt to certain niches, including inflamed or necrotic tissue during host infection.⁵² During hypoxia, several enzymes involved in ergosterol biosynthesis are transcriptionally elevated.⁵⁵ This might be due to the fact that oxygen is required in several steps of ergosterol biosynthesis including Cyp51 enzyme activity.⁵⁶ Decreased availability of the substrate oxygen is expected to decelerate the reaction speed of the involved enzymes. Increasing the amount of the enzymes would compensate for this to maintain the rate of ergosterol production when oxygen is rare. As CyB supports the Cyp51/Erg11 reaction kinetics,¹⁰ we addressed the question of whether CyB is also involved in this adaptation to hypoxia. We tested whether limited oxygen availability worsens the growth of $\Delta cybE$ strains with plate growth assays performed at low oxygen conditions (1% O₂, 5% CO₂). As a control under low oxygen conditions, we included a mutant strain ($\Delta srbA$) lacking SrpA, a transcription factor that is essential for adaptation to low oxygen conditions including the transcriptional activation of the ergosterol biosynthetic pathway.⁵⁵ In comparison to normoxic conditions, low oxygen conditions did not decrease the growth of the *cybE*-deficient strains, in contrast to $\Delta srbA$ (Fig. 2C and D). These data demonstrate that CyB is not essential during low oxygen conditions and that alternative electron-shuttling systems are also able to partially compensate for the loss of CyB under this condition.

Conclusion

This study illustrates that CyB assists Cyp51 enzyme activity in *A. fumigatus* and is consequently important for resistance to ergosterol biosynthesis pathway targeting antifungals. However, overexpression of *cybE* does not lead to increased resistance to these antifungals, making this enzyme an unlikely cause for resistance in clinical settings. Expression of *cybE* was found to be regulated by iron availability mediated by the transcription factor HapX. Recently, the CBC-HapX complex has been identified as a repressor of *cyp51A/erg11* gene expression in *A. fumigatus*.⁵⁴ Consequently, our data demonstrating the HapX-dependent iron regulation of *cybE* expression expand and underline the link between ergosterol biosynthesis, HapX and iron. Northern blot analysis revealed the compensation of CyB deficiency by the transcriptional upregulation of the alternative CPR system and the target gene *cyp51A*. Moreover, this study revealed remarkable differences between the two analysed *A. fumigatus* isolates, which highlights the extent to which different isolates of *A. fumigatus* can vary with respect to sterol homeostasis. This phenomenon has recently been observed regarding virulence and photoreponse in other *A. fumigatus* strains.^{57–59}

Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the joint D-A-CH program 'Novel molecular mechanisms of iron sensing and homeostasis in filamentous fungi' (Deutsche Forschungsgemeinschaft (DFG) DFG_HO 2596/1-1 to PH and Austrian Science Fund (FWF) I1346-B22 to HH). The funders had no role in study design, data collection and analysis; decision to publish; or preparation of the manuscript.

References

- 1 V. Burkina, M. K. Rasmussen, N. Pilipenko and G. Zamaratskaia, Comparison of xenobiotic-metabolising human, porcine, rodent, and piscine cytochrome P450, *Toxicology*, 2017, **375**, 10–27.
- 2 P. Durairaj, J.-S. Hur and H. Yun, Versatile biocatalysis of fungal cytochrome P450 monooxygenases, *Microb. Cell Fact.*, 2016, **15**, 125.
- 3 R. Bernhardt, Cytochromes P450 as versatile biocatalysts, *J. Biotechnol.*, 2006, **124**, 128–145.
- 4 I. G. Denisov, T. M. Makris, S. G. Sligar and I. Schlichting, Structure and Chemistry of Cytochrome P450, *Chem. Rev.*, 2004, **105**, 2253–2277.
- 5 L. Alcazar-Fuoli and E. Mellado, Ergosterol biosynthesis in *Aspergillus fumigatus*: Its relevance as an antifungal target and role in antifungal drug resistance, *Front. Microbiol.*, 2012, **3**, 439.
- 6 D. Werck-Reichhart and R. Feyereisen, Protein family review Cytochromes P450: a success story, *Genome Biol.*, 2000, **1**, 1–9.
- 7 K. J. McLean, M. Sabri, K. R. Marshall, R. J. Lawson, D. G. Lewis, D. Clift, P. R. Balding, A. J. Dunford, A. J. Warman, J. P. McVey, A.-M. Quinn, M. J. Sutcliffe, N. S. Scrutton and A. W. Munro, Biodiversity of cytochrome P450 redox systems, *Biochem. Soc. Trans.*, 2005, **33**, 796–801.
- 8 H. J. M. van den Brink, R. F. M. van Gorcom, C. A. M. J. J. van den Hondel and P. J. Punt, Cytochrome P450 Enzyme Systems in Fungi, *Fungal Genet. Biol.*, 1998, **23**, 1–17.
- 9 T. D. Porter, The roles of cytochrome *b*₅ in cytochrome P450 reactions, *J. Biochem. Mol. Toxicol.*, 2002, **16**, 311–316.
- 10 J. B. Schenkman and I. Jansson, The many roles of cytochrome *b*₅, *Pharmacol. Ther.*, 2003, **97**, 139–152.
- 11 P. Strittmatter, L. Spatz, D. Corcoran, M. J. Rogers, B. Setlow and R. Redline, Purification and properties of rat liver microsomal stearyl coenzyme A desaturase, *Proc. Natl. Acad. Sci. U. S. A.*, 1974, **71**, 4565–4569.
- 12 G. Vergères and L. Waskell, Cytochrome *b*₅, its functions, structure and membrane topology, *Biochimie*, 1995, **77**, 604–620.
- 13 D. C. Lamb, D. E. Kelly, N. J. Manning, M. A. Kaderbhai and S. L. Kelly, Biodiversity of the P450 catalytic cycle: Yeast cytochrome *b*₅/NADH cytochrome *b*₅ reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction, *FEBS Lett.*, 1999, **462**, 283–288.



- 14 G. Truan, J. C. Epinat, C. Rougeulle, C. Cullin and D. Pompon, Cloning and characterization of a yeast cytochrome *b₅*-encoding gene which suppresses ketoconazole hypersensitivity in a NADPH-P-450 reductase-deficient strain, *Gene*, 1994, **142**, 123–127.
- 15 K. Venkateswarlu, D. C. Lamb, D. E. Kelly, N. J. Manning and S. L. Kelly, The N-terminal membrane domain of yeast NADPH-cytochrome P450 (CYP) oxidoreductase is not required for catalytic activity in sterol biosynthesis or in reconstitution of CYP activity, *J. Biol. Chem.*, 1998, **273**, 4492–4496.
- 16 K. M. Rogers, C. A. Pierson, N. T. Culbertson, C. Mo, A. M. Sturm, J. Eckstein, R. Barbuch, N. D. Lees and M. Bard, Disruption of the *Candida albicans* CYB5 gene results in increased azole sensitivity, *Antimicrob. Agents Chemother.*, 2004, **48**, 3425–3435.
- 17 T. R. Sutter and J. C. Loper, Disruption of the *Saccharomyces cerevisiae* gene for NADPH-cytochrome P450 reductase causes increased sensitivity to ketoconazole, *Biochem. Biophys. Res. Commun.*, 1989, **160**, 1257–1266.
- 18 K. J. Kwon-Chung and J. A. Sugui, *Aspergillus fumigatus*-What Makes the Species a Ubiquitous Human Fungal Pathogen?, *PLoS Pathog.*, 2013, **9**, e1003743.
- 19 T. F. Patterson, G. R. Thompson, D. W. Denning, J. A. Fishman, S. Hadley, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, M. H. Nguyen, B. H. Segal, W. J. Steinbach, D. A. Stevens, T. J. Walsh, J. R. Wingard, J.-A. H. Young and J. E. Bennett, Executive Summary: Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America, *Clin. Infect. Dis.*, 2016, **63**, 433–442.
- 20 T. J. Walsh, E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. van Burik, J. R. Wingard and T. F. Patterson, Treatment of Aspergillosis: Clinical Practice Guidelines of the Infectious Diseases Society of America, *Clin. Infect. Dis.*, 2008, **46**, 327–360.
- 21 R. Garcia-Rubio, M. Cuenca-Estrella and E. Mellado, Triazole Resistance in *Aspergillus* Species: An Emerging Problem, *Drugs*, 2017, **77**, 599–613.
- 22 E. Mellado, T. M. Diaz-Guerra, M. Cuenca-Estrella and J. L. Rodriguez-Tudela, Identification of two different 14- α sterol demethylase-related genes (*cyp51A* and *cyp51B*) in *Aspergillus fumigatus* and other *Aspergillus* species, *J. Clin. Microbiol.*, 2001, **39**, 2431–2438.
- 23 E. Mellado, G. Garcia-Effron, M. J. Buitrago, L. Alcazar-Fuoli, M. Cuenca-Estrella and J. L. Rodriguez-Tudela, Targeted Gene Disruption of the 14-Sterol Demethylase (*cyp51A*) in *Aspergillus fumigatus* and Its Role in Azole Drug Susceptibility, *Antimicrob. Agents Chemother.*, 2005, **49**, 2536–2538.
- 24 W. Hu, S. Sillaots, S. Lemieux, J. Davison, S. Kauffman, A. Breton, A. Linteau, C. Xin, J. Bowman, J. Becker, B. Jiang and T. Roemer, Essential Gene Identification and Drug Target Prioritization in *Aspergillus fumigatus*, *PLoS Pathog.*, 2007, **3**, e24.
- 25 L. Alcazar-Fuoli and E. Mellado, Current status of antifungal resistance and its impact on clinical practice, *Br. J. Haematol.*, 2014, **166**, 471–484.
- 26 J. Park, S. Lee, J. Choi, K. Ahn, B. Park, J. Park, S. Kang and Y.-H. Lee, Fungal cytochrome P450 database, *BMC Genomics*, 2008, **9**, 402.
- 27 L. M. Podust, D. H. Sherman, F. H. Arnold, B. Tudzynski, R. A. E. Butchko, M. Kimura, B. Youn, B. S. Kim, J. S. Ahn, D. H. Sherman, Y. J. Yoon, M. J. Sutcliffe, N. S. Scrutton, A. W. Munro, J. R. Falck, T. Shimada, M. R. Waterman, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill and D. A. Hopwood, Diversity of P450 enzymes in the biosynthesis of natural products, *Nat. Prod. Rep.*, 2012, **29**, 1251.
- 28 H. Haas, Iron – A Key Nexus in the Virulence of *Aspergillus fumigatus*, *Front. Microbiol.*, 2012, **3**, 28.
- 29 F. Gsaller, P. Hortschansky, S. R. Beattie, V. Klammer, K. Tuppatsch, B. E. Lechner, N. Rietzschel, E. R. Werner, A. A. Vogan, D. Chung, M. Kato, R. A. Cramer, A. A. Brakhage and H. Haas, The Janus transcription factor HapX controls fungal adaptation to both iron starvation and iron excess, *EMBO J.*, 2014, **33**, 2261–2276.
- 30 M. Schrettl and H. Haas, Iron homeostasis-Achilles' heel of *Aspergillus fumigatus*?, *Curr. Opin. Microbiol.*, 2011, **14**, 400–405.
- 31 M. Blatzer, B. M. Barker, S. D. Willger, N. Beckmann, S. J. Blosser, E. J. Cornish, A. Mazurie, N. Grahl, H. Haas and R. a Cramer, SREBP coordinates iron and ergosterol homeostasis to mediate triazole drug and hypoxia responses in the human fungal pathogen *Aspergillus fumigatus*, *PLoS Genet.*, 2011, **7**, e1002374.
- 32 G. Pontecorvo, J. A. Roper, L. M. Hemmons, K. D. Macdonald and A. W. J. Bufton, The genetics of *Aspergillus nidulans*, *Adv. Genet.*, 1953, **5**, 141–238.
- 33 M. G. Fraczek, M. Bromley, A. Buied, C. B. Moore, R. Rajendran, R. Rautemaa, G. Ramage, D. W. Denning and P. Bowyer, The *cdr1B* efflux transporter is associated with non-*cyp51a*-mediated itraconazole resistance in *Aspergillus fumigatus*, *J. Antimicrob. Chemother.*, 2013, **68**, 1486–1496.
- 34 S. Krappmann, N. Jung, B. Medic, S. Busch, R. A. Prade and G. H. Braus, The *Aspergillus nidulans* F-box protein GrrA links SCF activity to meiosis, *Mol. Microbiol.*, 2006, **61**, 76–88.
- 35 I. Zadra, B. Abt, W. Parson and H. Haas, xylP promoter-based expression system and its use for antisense downregulation of the *Penicillium chrysogenum* nitrogen regulator NRE, *Appl. Environ. Microbiol.*, 2000, **66**, 4810–4816.
- 36 T. Hartmann, M. Dümig, B. M. Jaber, E. Szewczyk, P. Olbermann, J. Morschhäuser and S. Krappmann, Validation of a self-excising marker in the human pathogen *Aspergillus fumigatus* by employing the beta-rec/six site-specific recombination system, *Appl. Environ. Microbiol.*, 2010, **76**, 6313–6317.
- 37 L. Alcazar-Fuoli, E. Mellado, G. Garcia-Effron, J. F. Lopez, J. O. Grimalt, J. M. Cuenca-Estrella and J. L. Rodriguez-Tudela, Ergosterol biosynthesis pathway in *Aspergillus fumigatus*, *Steroids*, 2008, **73**, 339–347.

- 38 C. Müller, V. Staudacher, J. Krauss, M. Giera and F. Bracher, A convenient cellular assay for the identification of the molecular target of ergosterol biosynthesis inhibitors and quantification of their effects on total ergosterol biosynthesis, *Steroids*, 2013, **78**, 483–493.
- 39 P. Keller, C. Müller, I. Engelhardt, E. Hiller, K. Lemuth, H. Eickhoff, K.-H. Wiesmüller, A. Burger-Kentscher, F. Bracher and S. Rupp, An Antifungal Benzimidazole Derivative Inhibits Ergosterol Biosynthesis and Reveals Novel Sterols, *Antimicrob. Agents Chemother.*, 2015, **59**, 6296–6307.
- 40 C. Müller and F. Bracher, Determination by GC-IT/MS of phytosterols in herbal medicinal products for the treatment of lower urinary tract symptoms and food products marketed in Europe, *Planta Med.*, 2015, **81**, 613–620.
- 41 A. G. S. Warrilow, J. E. Parker, C. L. Price, W. D. Nes, S. L. Kelly and D. E. Kelly, *In Vitro* Biochemical Study of CYP51-Mediated Azole Resistance in *Aspergillus fumigatus*, *Antimicrob. Agents Chemother.*, 2015, **59**, 7771–7778.
- 42 A. K. Gupta, M. Paquet and F. C. Simpson, Therapies for the treatment of onychomycosis, *Clin. Dermatol.*, 2013, **31**, 544–554.
- 43 T. D. Porter, Electron Transfer Pathways in Cholesterol Synthesis, *Lipids*, 2015, **50**, 927–936.
- 44 K. B. Boyle, L. R. Stephens and P. T. Hawkins, Activation of the neutrophil NADPH oxidase by *Aspergillus fumigatus*, *Ann. N. Y. Acad. Sci.*, 2012, **1273**, 68–73.
- 45 T. Finkel and N. J. Holbrook, Oxidants, oxidative stress and the biology of ageing, *Nature*, 2000, **408**, 239–247.
- 46 M. Schrettl, N. Beckmann, J. Varga, T. Heinekamp, I. D. Jacobsen, C. Jöchl, T. a Moussa, S. Wang, F. Gsaller, M. Blatzer, E. R. Werner, W. C. Niermann, A. a Brakhage and H. Haas, HapX-mediated adaption to iron starvation is crucial for virulence of *Aspergillus fumigatus*, *PLoS Pathog.*, 2010, **6**, e1001124.
- 47 P. Hortschansky, M. Eisendle, Q. Al-Abdallah, A. D. Schmidt, S. Bergmann, M. Thön, O. Kniemeyer, B. Abt, B. Seeber, E. R. Werner, M. Kato, A. a Brakhage and H. Haas, Interaction of HapX with the CCAAT-binding complex—a novel mechanism of gene regulation by iron, *EMBO J.*, 2007, **26**, 3157–3168.
- 48 H. Haas, M. Schoeser, E. Lesuisse, J. F. Ernst, W. Parson, B. Abt, G. Winkelman and H. Oberegger, Characterization of the *Aspergillus nidulans* transporters for the siderophores enterobactin and triacetylfusarinine C, *Biochem. J.*, 2003, **371**, 505–513.
- 49 F. Gsaller, M. Eisendle, B. E. Lechner, M. Schrettl, H. Lindner, D. Müller, S. Geley and H. Haas, The interplay between vacuolar and siderophore-mediated iron storage in *Aspergillus fumigatus*, *Metallomics*, 2012, **4**, 1262–1270.
- 50 A. Buied, C. B. Moore, D. W. Denning and P. Bowyer, High-level expression of cyp51B in azole-resistant clinical *Aspergillus fumigatus* isolates, *J. Antimicrob. Chemother.*, 2013, **68**, 512–514.
- 51 C. M. Bien and P. J. Espenshade, Sterol regulatory element binding proteins in fungi: Hypoxic transcription factors linked to pathogenesis, *Eukaryotic Cell*, 2010, **9**, 352–359.
- 52 N. Grahl, K. M. Shepardson, D. Chung and R. A. Cramer, Hypoxia and fungal pathogenesis: to air or not to air?, *Eukaryotic Cell*, 2012, **11**, 560–570.
- 53 S. J. Blosser and R. A. Cramer, SREBP-dependent triazole susceptibility in *Aspergillus fumigatus* is mediated through direct transcriptional regulation of erg11A (cyp51A), *Antimicrob. Agents Chemother.*, 2012, **56**, 248–257.
- 54 F. Gsaller, P. Hortschansky, T. Furukawa, P. D. Carr, B. Rash, J. Capilla, C. Müller, F. Bracher, P. Bowyer, H. Haas, A. A. Brakhage and M. J. Bromley, Sterol Biosynthesis and Azole Tolerance Is Governed by the Opposing Actions of SrbA and the CCAAT Binding Complex, *PLoS Pathog.*, 2016, **12**, e1005775.
- 55 D. Chung, B. M. Barker, C. C. Carey, B. Merriman, E. R. Werner, B. E. Lechner, S. Dhingra, C. Cheng, W. Xu, S. J. Blosser, K. Morohashi, A. Mazurie, T. K. Mitchell, H. Haas, A. P. Mitchell and R. A. Cramer, ChIP-seq and In Vivo Transcriptome Analyses of the *Aspergillus fumigatus* SREBP SrbA Reveals a New Regulator of the Fungal Hypoxia Response and Virulence, *PLoS Pathog.*, 2014, **10**, e1004487.
- 56 A. M. Galea and A. J. Brown, Special relationship between sterols and oxygen: Were sterols an adaptation to aerobic life?, *Free Radical Biol. Med.*, 2009, **47**, 880–889.
- 57 N. P. Keller, Heterogeneity Confounds Establishment of ‘a’ Model Microbial Strain, *mBio*, 2017, **8**, e00135.
- 58 C. H. Kowalski, S. R. Beattie, K. K. Fuller, E. A. McGurk, Y.-W. Tang, T. M. Hohl, J. J. Obar and R. A. Cramer, Heterogeneity among Isolates Reveals that Fitness in Low Oxygen Correlates with *Aspergillus fumigatus* Virulence, *mBio*, 2016, **7**, e01515.
- 59 K. K. Fuller, R. A. Cramer, M. E. Zegans, J. C. Dunlap and J. J. Loros, *Aspergillus fumigatus* Photobiology Illuminates the Marked Heterogeneity between Isolates, *mBio*, 2016, **7**, e01517.

